



## 1. Type of Application

This new application is for a(n)

(check one applicable item below)

- Original (nonprovisional)
- Design
- Plant

**WARNING:** *Do not use this transmittal for a completion in the U.S. of an International Application under 35 U.S.C. § 371(c)(4), unless the International Application is being filed as a divisional, continuation or continuation-in-part application.*

**WARNING:** *Do not use this transmittal for the filing of a provisional application.*

**NOTE:** *If one of the following 3 items apply, then complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF A PRIOR U.S. APPLICATION CLAIMED and a NOTIFICATION IN PARENT APPLICATION OF THE FILING OF THIS CONTINUATION APPLICATION.*

- Divisional.
- Continuation.
- Continuation-in-part (C-I-P).

## 2. Benefit of Prior U.S. Application(s) (35 U.S.C. §§ 119(e), 120, or 121)

**NOTE:** *A nonprovisional application may claim an invention disclosed in one or more prior filed copending nonprovisional applications or copending international applications designating the United States of America. In order for a nonprovisional application to claim the benefit of a prior filed copending nonprovisional application or copending international application designating the United States of America, each prior application must name as an inventor at least one inventor named in the later filed nonprovisional application and disclose the named inventor's invention claimed in at least one claim of the later filed nonprovisional application in the manner provided by the first paragraph of 35 U.S.C. § 112. Each prior application must also be:*

- (i) An international application entitled to a filing date in accordance with PCT Article 11 and designating the United States of America; or*
- (ii) Complete as set forth in § 1.51(b); or*
- (iii) Entitled to a filing date as set forth in § 1.53(b) or § 1.53(d) and include the basic filing fee set forth in § 1.16; or*
- (iv) Entitled to a filing date as set forth in § 1.53(b) and have paid therein the processing and retention fee set forth in § 1.21(l) within the time period set forth in § 1.53(f).*

*37 C.F.R. § 1.78(a)(1).*

**NOTE:** *If the new application being transmitted is a divisional, continuation or a continuation-in-part of a parent case, or where the parent case is an International Application which designated the U.S., or benefit of a prior provisional application is claimed, then check the following item and complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.*

**WARNING:** *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

(New Application Transmittal [4-1]—page 2 of 11)

**WARNING:** When the last day of pendency of a provisional application falls on a Saturday, Sunday, or Federal holiday within the District of Columbia, any nonprovisional application claiming benefit of the provisional application must be filed prior to the Saturday, Sunday, or Federal holiday within the District of Columbia. See 37 C.F.R. § 1.78(a)(3).

- The new application being transmitted claims the benefit of prior U.S. application(s). Enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

### 3. Papers Enclosed

**A.** Required for filing date under 37 C.F.R. § 1.53(b) (Regular) or 37 C.F.R. § 1.153 (Design) Application

66 Pages of specification (includes cover page)

3 Pages of claims

15 Sheets of drawing

**WARNING:** DO NOT submit original drawings. A high quality copy of the drawings should be supplied when filing a patent application. The drawings that are submitted to the Office must be on strong, white, smooth, and non-shiny paper and meet the standards according to § 1.84. If corrections to the drawings are necessary, they should be made to the original drawing and a high-quality copy of the corrected original drawing then submitted to the Office. Only one copy is required or desired. For comments on proposed then-new 37 C.F.R. § 1.84, see Notice of March 9, 1988 (1990 O.G. 57-62).

**NOTE:** "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page . . ." 37 C.F.R. § 1.84(c).

(complete the following, if applicable)

- The enclosed drawing(s) are photograph(s), and there is also attached a "PETITION TO ACCEPT PHOTOGRAPH(S) AS DRAWING(S)." 37 C.F.R. § 1.84(b).  
 formal  
 informal

**B. Other Papers Enclosed**

3 Pages of declaration and power of attorney

1 Pages of abstract

1 Other Statement re: Sequence Listing

### 4. Additional papers enclosed

Amendment to claims

- Cancel in this applications claims \_\_\_\_\_ before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)  
 Add the claims shown on the attached amendment. (Claims added have been numbered consecutively following the highest numbered original claims.)

Preliminary Amendment

Information Disclosure Statement (37 C.F.R. § 1.98)

Form PTO-1449 (PTO/SB/08A and 08B)

Citations

- Declaration of Biological Deposit
- Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence.
- Authorization of Attorney(s) to Accept and Follow Instructions from Representative
- Special Comments
- Other Papercopy of sequence listing

#### 5. Declaration or oath (including power of attorney)

NOTE: A newly executed declaration is not required in a continuation or divisional application provided that the prior nonprovisional application contained a declaration as required, the application being filed is by all or fewer than all the inventors named in the prior application, there is no new matter in the application being filed, and a copy of the executed declaration filed in the prior application (showing the signature or an indication thereon that it was signed) is submitted. The copy must be accompanied by a statement requesting deletion of the names of person(s) who are not inventors of the application being filed. If the declaration in the prior application was filed under § 1.47, then a copy of that declaration must be filed accompanied by a copy of the decision granting § 1.47 status or, if a nonsigning person under § 1.47 has subsequently joined in a prior application, then a copy of the subsequently executed declaration must be filed. See 37 C.F.R. §§ 1.63(d)(1)-(3).

NOTE: A declaration filed to complete an application must be executed, identify the specification to which it is directed, identify each inventor by full name including family name and at least one given name, without abbreviation together with any other given name or initial, and the residence, post office address and country or citizenship of each inventor, and state whether the inventor is a sole or joint inventor. 37 C.F.R. § 1.63(a)(1)-(4).

NOTE: "The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.62, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(i) is filed supplying or changing the name or names of the inventor or inventors." 37 C.F.R. § 1.41(a)(1).

- Enclosed

Executed by

(check all applicable boxes)

- inventor(s).
- legal representative of inventor(s).  
37 C.F.R. §§ 1.42 or 1.43.
- joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.
- This is the petition required by 37 C.F.R. § 1.47 and the statement required by 37 C.F.R. § 1.47 is also attached. See item 13 below for fee.

- Not Enclosed.

NOTE: Where the filing is a completion in the U.S. of an International Application or where the completion of the U.S. application contains subject matter in addition to the International Application, the application may be treated as a continuation or continuation-in-part, as the case may be, utilizing ADDED PAGE FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION CLAIMED.

- Application is made by a person authorized under 37 C.F.R. § 1.41(c) on behalf of all the above named inventor(s).

(The declaration or oath, along with the surcharge required by 37 C.F.R. § 1.16(e) can be filed subsequently).

- Showing that the filing is authorized.  
(not required unless called into question. 37 C.F.R. § 1.41(d))

#### 6. Inventorship Statement

**WARNING:** If the named inventors are each not the inventors of all the claims an explanation, including the ownership of the various claims at the time the last claimed invention was made, should be submitted.

The inventorship for all the claims in this application are:

- The same.

or

- Not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made,  
 is submitted.  
 will be submitted.

#### 7. Language

**NOTE:** An application including a signed oath or declaration may be filed in a language other than English. An English translation of the non-English language application and the processing fee of \$130.00 required by 37 C.F.R. § 1.17(k) is required to be filed with the application, or within such time as may be set by the Office. 37 C.F.R. § 1.52(d).

- English

- Non-English

- The attached translation includes a statement that the translation is accurate. 37 C.F.R. § 1.52(d).

#### 8. Assignment

- An assignment of the invention to New England Biolabs, Inc.

32 Tozer Road; Beverly, MA 01915

- is attached. A separate  "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or  FORM PTO 1595 is also attached.  
 will follow.

**NOTE:** "If an assignment is submitted with a new application, send two separate letters-one for the application and one for the assignment." Notice of May 4, 1990 (1114 O.G. 77-78).

**WARNING:** A newly executed "CERTIFICATE UNDER 37 C.F.R. § 3.73(b)" must be filed when a continuation-in-part application is filed by an assignee. Notice of April 30, 1993, 1150 O.G. 62-64.

(New Application Transmittal [4-1]—page 5 of 11)

**9. Certified Copy**

Certified copy(ies) of application(s)

Country	Appln. No.	Filed
Country	Appln. No.	Filed
Country	Appln. No.	Filed

from which priority is claimed

- is (are) attached.  
 will follow.

NOTE: *The foreign application forming the basis for the claim for priority must be referred to in the oath or declaration. 37 C.F.R. § 1.55(a) and 1.63.*

NOTE: *This item is for any foreign priority for which the application being filed directly relates. If any parent U.S. application or International Application from which this application claims benefit under 35 U.S.C. § 120 is itself entitled to priority from a prior foreign application, then complete item 18 on the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.*

**10. Fee Calculation (37 C.F.R. § 1.16)****A.  Regular application**

CLAIMS AS FILED					
Number filed	Number Extra	Rate	Basic Fee 37 C.F.R. § 1.16(a)		
			\$690.00		
<b>Total</b>					
Claims (37 C.F.R. § 1.16(c))	17	- 20 = 0	× \$ 18.00	0.00	
<b>Independent</b>					
Claims (37 C.F.R. § 1.16(b))	4	- 3 = 1	× \$ 78.00	80.00	
Multiple dependent claim(s), if any (37 C.F.R. § 1.16(d))	Yes		+ \$260.00	270.00	

- Amendment cancelling extra claims is enclosed.  
 Amendment deleting multiple-dependencies is enclosed.  
 Fee for extra claims is not being paid at this time.

NOTE: *If the fees for extra claims are not paid on filing they must be paid or the claims cancelled by amendment, prior to the expiration of the time period set for response by the Patent and Trademark Office in any notice of fee deficiency. 37 C.F.R. § 1.16(d).*

Filing Fee Calculation \$ 1060.00

**B.  Design application**

(\$310.00—37 C.F.R. § 1.16(f))

Filing Fee Calculation \$ \_\_\_\_\_

(New Application Transmittal [4-1]—page 6 of 11)

C.  Plant application  
(\$480.00—37 C.F.R. § 1.16(g))

Filing fee calculation \$ \_\_\_\_\_

**11. Small Entity Statement(s)**

- Statement(s) that this is a filing by a small entity under 37 C.F.R. § 1.9 and 1.27 is (are) attached.

**WARNING:** "Status as a small entity must be specifically established in each application or patent in which the status is available and desired. Status as a small entity in one application or patent does not affect any other application or patent, including applications or patents which are directly or indirectly dependent upon the application or patent in which the status has been established. The refiling of an application under § 1.53 as a continuation, division, or continuation-in-part (including a continued prosecution application under § 1.53(d)), or the filing of a reissue application requires a new determination as to continued entitlement to small entity status for the continuing or reissue application. A nonprovisional application claiming benefit under 35 U.S.C. § 119(e), 120, 121, or 365(c) of a prior application, or a reissue application may rely on a statement filed in the prior application or in the patent if the nonprovisional application or the reissue application includes a reference to the statement in the prior application or in the patent or includes a copy of the statement in the prior application or in the patent and status as a small entity is still proper and desired. The payment of the small entity basic statutory filing fee will be treated as such a reference for purposes of this section." 37 C.F.R. § 1.28(a)(2).

**WARNING:** "Small entity status must not be established when the person or persons signing the . . . statement can unequivocally make the required self-certification." M.P.E.P., § 509.03, 6th ed., rev. 2, July 1996 (emphasis added).

(complete the following, if applicable)

- Status as a small entity was claimed in prior application

\_\_\_\_\_ / \_\_\_\_\_, filed on \_\_\_\_\_, from which benefit is being claimed for this application under:

- 35 U.S.C. §  119(e),  
 120,  
 121,  
 365(c),

and which status as a small entity is still proper and desired.

- A copy of the statement in the prior application is included.

Filing Fee Calculation (50% of A, B or C above)

\$ 530.00

**NOTE:** Any excess of the full fee paid will be refunded if small entity status is established and a refund request are filed within 2 months of the date of timely payment of a full fee. The two-month period is not extendable under § 1.136. 37 C.F.R. § 1.28(a).

**12. Request for International-Type Search (37 C.F.R. § 1.104(d))**

(complete, if applicable)

- Please prepare an international-type search report for this application at the time when national examination on the merits takes place.

**13. Fee Payment Being Made at This Time**

- Not Enclosed  
 No filing fee is to be paid at this time.  
*(This and the surcharge required by 37 C.F.R. § 1.16(e) can be paid subsequently.)*
- Enclosed
- |   |                  |
|---|------------------|
| <input checked="" type="checkbox"/> Filing fee  | \$ <u>530.00</u> |
| <input checked="" type="checkbox"/> Recording assignment<br>(\$40.00; 37 C.F.R. § 1.21(h))<br>(See attached "COVER SHEET FOR<br>ASSIGNMENT ACCOMPANYING NEW<br>APPLICATION".)   | \$ <u>40.00</u>  |
| <input type="checkbox"/> Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached<br>(\$130.00; 37 C.F.R. §§ 1.47 and 1.17(i)) | \$ _____         |
| <input type="checkbox"/> For processing an application with a specification in a non-English language<br>(\$130.00; 37 C.F.R. §§ 1.52(d) and 1.17(k))   | \$ _____         |
| <input type="checkbox"/> Processing and retention fee<br>(\$130.00; 37 C.F.R. §§ 1.53(d) and 1.21(l))   | \$ _____         |
| <input type="checkbox"/> Fee for international-type search report<br>(\$40.00; 37 C.F.R. § 1.21(e))   | \$ _____         |

NOTE: 37 C.F.R. § 1.21(l) establishes a fee for processing and retaining any application that is abandoned for failing to complete the application pursuant to 37 C.F.R. § 1.53(f) and this, as well as the changes to 37 C.F.R. §§ 1.53 and 1.78(a)(1), indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid, or the processing and retention fee of § 1.21(l) must be paid, within 1 year from notification under § 53(f).

Total fees enclosed \$ 570.00

**14. Method of Payment of Fees**

- Check in the amount of \$ 570.00
- Charge Account No. \_\_\_\_\_ in the amount of  
\$ \_\_\_\_\_.

A duplicate of this transmittal is attached.

NOTE: Fees should be itemized in such a manner that it is clear for which purpose the fees are paid. 37 C.F.R. § 1.22(b).

## **15. Authorization to Charge Additional Fees**

**WARNING:** If no fees are to be paid on filing, the following items should not be completed.

**WARNING:** Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges, if extra claim charges are authorized.

- The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No. 14-0740.
- 37 C.F.R. § 1.16(a), (f) or (g) (filing fees)
- 37 C.F.R. § 1.16(b), (c) and (d) (presentation of extra claims)

**NOTE:** Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

- 37 C.F.R. § 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)
- 37 C.F.R. § 1.17(a)(1)–(5) (extension fees pursuant to § 1.136(a)).
- 37 C.F.R. § 1.17 (application processing fees)

**NOTE:** ". . . A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

- 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

**NOTE:** Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

**NOTE:** 37 C.F.R. § 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying, . . . the issue fee. . ." From the wording of 37 C.F.R. § 1.28(b), (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

(New Application Transmittal [4-1]—page 9 of 11)

#### **16. Instructions as to Overpayment**

**NOTE:** "...Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

- Credit Account No. 14-0740
- Refund

Reg. No. 30901

Tel. No. (978) 927-5054 X:292

Customer No.

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**SIGNATURE OF PRACTITIONER**

Gregory D. Williams  
General Counsel

(type or print name of attorney)

New England Biolabs, Inc.  
32 Tozer Road

P.O. Address

Beverly, MA 01915

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(New Application Transmittal [4-1]—page 10 of 11)

**Incorporation by reference of added pages**

(check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED)

- Plus Added Pages for New Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed

Number of pages added \_\_\_\_\_

- Plus Added Pages for Papers Referred to in Item 4 Above

Number of pages added \_\_\_\_\_

- Plus added pages deleting names of inventor(s) named in prior application(s) who is/are no longer inventor(s) of the subject matter claimed in this application.

Number of pages added \_\_\_\_\_

- Plus "Assignment Cover Letter Accompanying New Application"

Number of pages added \_\_\_\_\_

 **Statement Where No Further Pages Added**

(if no further pages form a part of this Transmittal, then end this Transmittal with this page and check the following item)

- This transmittal ends with this page.

(New Application Transmittal [4-1]—page 11 of 11)

Applicant Vaisvila, et al.       Patentee \_\_\_\_\_  
 Application No.       Patent No. \_\_\_\_\_  
 Filed on \_\_\_\_\_       Issued on \_\_\_\_\_  
Title: Method For Cloning And Producing The MseI Restriction Endonuclease

**STATEMENT CLAIMING SMALL ENTITY STATUS  
(37 CFR 1.9(f) and 1.27(c))—SMALL BUSINESS CONCERN**

I hereby state that I am

- the owner of the small business concern identified below;  
 an official of the small business concern empowered to act on behalf of the concern identified below:

Name of Small Business Concern New England Biolabs, Inc.

Address of Small Business Concern 32 Tozer Road  
Beverly, MA 01915

I hereby state that the above identified small business concern qualifies as a small business concern, as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office under Sections 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby state that rights under contract or law have been conveyed to, and remain with, the small business concern identified above, with regard to the invention described in

- the specification filed herewith, with title as listed above.  
 the application identified above.  
 the patent identified above.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below\* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c), if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

\*NOTE: Separate statements are required from each named person, concern or organization having rights to the invention as to their status as small entities. (37 CFR 1.27)

(Small Entity-Small Business [7-4]—page 1 of 2)

Each such person, concern or organization having any rights in the invention is listed below:

- No such person, concern, or organization exists.  
 Each such person, concern or organization is listed below.

Name New England Biolabs, Inc.

Address 32 Tozer Road

Beverly, MA 01915

INDIVIDUAL       SMALL BUSINESS CONCERN       NONPROFIT ORGANIZATION

Name \_\_\_\_\_

Address \_\_\_\_\_

INDIVIDUAL       SMALL BUSINESS CONCERN       NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small business entity is no longer appropriate. (37 CFR 1.28(b))

(check the following item, if desired)

NOTE: The following verification statement need not be made in accordance with the rules published on Oct. 10, 1997, 62 Fed. Reg. 52,131, effective Dec. 1, 1997.

NOTE: "The presentation to the Office (whether by signing, filing, submitting, or later advocating) of any paper by a party, whether a practitioner or non-practitioner, constitutes a certification under § 10.18(b) of this chapter. Violations of § 10.18(b)(2) of this chapter by a party, whether a practitioner or non-practitioner, may result in the imposition of sanctions under § 10.18(c) of this chapter. Any practitioner violating § 10.18(b) may also be subject to disciplinary action. See §§ 10.18(d) and 10.23(c)(15)." 37 C.F.R. § 1.4(d)(2).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing Gregory D. Williams

Title of Person if Other Than Owner Assistant Clerk

Address of Person Signing 32 Tozer Road

Beverly, MA 01915

SIGNATURE [Signature]

Date 10/12/02

Docket No. NEB-181

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
APPLICATION FOR UNITED STATES LETTERS PATENT

**INVENTOR (S):** Romualdas Vaisvila  
Richard D. Morgan  
Rebecca B. Kucera  
Toby E. Claus  
Elisabeth A. Raleigh

**TITLE:** METHOD FOR CLONING AND PRODUCING  
THE *MseI* RESTRICTION ENDONUCLEASE

**ATTORNEY:** Gregory D. Williams  
General Counsel  
NEW ENGLAND BIOLABS INC.,  
32 Tozer Road  
Beverly, Massachusetts 01915  
(978) 927-5054; Ext. 292

**EXPRESS MAILING LABEL NO.:** EK589681297US

**METHOD FOR CLONING AND PRODUCING THE MseI  
RESTRICTION ENDONUCLEASE**

**BACKGROUND OF THE INVENTION**

5

Restriction endonucleases belong to the class of enzymes called nucleases which degrade or cut single or double stranded DNA. A restriction endonuclease acts by recognizing and binding to particular sequences of nucleotides (the 'recognition sequence') along the DNA molecule. Once bound, the endonuclease cleaves the molecule within or to one side of the recognition sequence. The location of cleavage may differ among various restriction endonucleases, though for any given endonuclease the position is fixed. Different restriction endonucleases have different affinity for recognition sequences. More than two hundred restriction endonucleases recognizing unique specificities have been identified among thousands of bacterial and archaeal species that have been examined to date.

20

Restriction endonucleases are classified on the basis of their composition and cofactor requirements, the nature of target sequence, and the position of the site of DNA cleavage with respect to the target sequence (Yuan, R. *Ann. Rev. Biochem.*, 50:285-315 (1981)). Currently three distinct, well-characterized classes of restriction endonucleases are known (I, II and III). Type I enzymes recognize specific sequences,

but cleave randomly with respect to that sequence. The type III restriction endonucleases recognize specific sequences, cleave at a defined position to one side of that sequence, but never give complete digestion. Neither of these two kinds of enzymes is suitable for practical use. The type II restriction endonucleases recognize specific sequences (4-8 nucleotides long) and cleave at a defined position either within or very close to that sequence. Usually they require only Mg<sup>2+</sup> ions for their action. When they are purified away from other bacterial components, type II restriction endonucleases can be used in the laboratory to cleave DNA molecules into specific fragments. This property allows the researcher to manipulate the DNA molecule and analyze the resulting constructions.

Bacteria tend to possess at most, only a small number of restriction endonucleases per isolate. The restriction endonucleases are designated by a three-letter acronym derived from the name of organism in which they occur (Smith and Nathans, *J. Mol. Biol.* 81:419-423 (1973)). The first letter comes from the genus, and the second and third letters come from the species. Thus, a strain of the species *Deinococcus radiophilus* for example, synthesizes three different type II restriction endonucleases, named *DraI*, *DraII* and *DraIII*. These enzymes recognize and cleave the sequences TTTAAA, PuGGNCCPy and CACNNNGTG, respectively. *Escherichia coli* RY13, on the other hand, synthesizes only one type II restriction enzyme, *EcoRI*, which recognizes the sequence

DOCKET NO. NEB-181, FILED IN THE U.S. COURT OF APPEALS FOR THE FEDERAL CIRCUIT

GAATTC (Roberts R.J and Macelis D., *Nucl. Acids Res.*, 28:306-7 (2000)).

A second component of bacterial and archaeal restriction systems are the modification methylases (Roberts and Halford, in 'Nucleases', 2<sup>nd</sup> ed., Linn et al., ed.'s, p. 35-88 (1993)). These enzymes are complementary to restriction endonucleases and they provide the means by which bacteria are able to protect their own DNA and distinguish it from foreign, invading DNA. Modification methylases recognize and bind to the same recognition sequence as the corresponding restriction endonuclease, but instead of cleaving the DNA, they chemically modify one or other of the nucleotides within the sequence by the addition of a methyl group. Following methylation, the recognition sequence is no longer cleaved by the restriction endonuclease. The DNA of a bacterial cell is modified by virtue of the activity of its modification methylase, and is therefore insensitive to the presence of the endogenous restriction endonuclease. It is only unmodified, and therefore identifiably foreign DNA, that is sensitive to restriction endonuclease recognition and cleavage.

It is thought that in nature, type II restriction endonucleases cleave foreign DNA such as viral and plasmid DNA when this DNA has not been modified by the appropriate modification enzyme (Wilson and Murray, *Annu. Rev. Genet.* 25:585-627 (1991)). In this way, cells are protected from invasion by foreign DNA. Thus, it has been widely believed

that evolution of type II restriction modification systems has been driven by the cell's need to protect itself from infection by foreign DNA (the cellular defense hypothesis).

5           With the advent of genetic engineering technology, it is now possible to clone genes and to produce the proteins and enzymes that they encode in greater quantities than are obtainable by conventional purification techniques. The key to isolating clones of restriction endonuclease genes is to  
10          develop a simple and reliable method to identify such clones within gene libraries. One potential difficulty is that some restriction endonuclease and methylase genes may not express in *E. coli* due to differences in the transcriptional and translational machinery of the source organism and of *E. coli*, such as differences in promotor or ribosome binding sites or  
15          the codon composition of the gene. The isolation of the methylase gene requires that the methylase express well enough in *E. coli* to fully protect at least some of the plasmids carrying the gene. The isolation of the endonuclease in active form requires that the methylase express well enough to  
20          protect the host DNA fully, or at least enough to prevent lethal damage from cleavage by the endonuclease. Another obstacle to cloning restriction-modification systems lies in the discovery that some strains of *E. coli* react adversely to cytosine or adenine modification; they possess systems that  
25          destroy DNA containing methylated cytosine (Raleigh and Wilson, *Proc. Natl. Acad. Sci., USA* 83:9070-9074, (1986)), or methylated adenine (Heitman and Model, *J. Bact.* 196:3243-

3250, (1987)); Raleigh, et al., *Genetics*, 122:279-296, (1989))

Waite-Rees, et al., *J. Bacteriology*, 173:5207-5219 (1991)).

Cytosine-specific or adenine-specific methylase genes cannot be cloned easily into these strains, either on their own, or together with their corresponding endonuclease genes. To avoid this problem it is necessary to use mutant strains of *E. coli* (*McrA*<sup>-</sup> and *McrB*<sup>-</sup> or *Mrr*<sup>-</sup>) in which these systems are defective.

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Several approaches have been used to clone restriction genes into *E. coli*:

1) Selection based on phage restriction

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The first cloned systems used bacteriophage infection as a means of identifying or selecting restriction endonuclease clones (*EcoRII*: Kosykh et al., *Molec. Gen. Genet.* 178:717-719, (1980)); *HhaII*: Mann et al., *Gene* 3:97-112, (1978)); *PstI*: Walder et al., *Proc. Nat Acad. Sci.* 78:1503-1507,

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(1981)). Since the presence of restriction-modification systems in bacteria enable them to resist infection by bacteriophages, cells that carry cloned restriction-modification genes can, in principle, be selectively isolated as survivors from libraries that have been exposed to phage. This method

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resistance to confer selective survival under standard conditions.

2) Selection based on vector modification

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A second approach which is being used to clone a growing number of systems, involves selection for an active methylase gene (refer to U.S. Pat. No. 5,200,333 and *BsuRI*: Kiss et al., *Nucl. Acid. Res.* 13:6403-6421, (1985)). Since restriction and modification genes are often closely linked, both genes can often be cloned simultaneously. This selection does not always yield a complete restriction system however, but instead may yield only the methyltransferase gene (*BspRI*: Szomolanyi et al., *Gene* 10:219-225, (1980); *BcnI*: Janulaitis et al, *Gene* 20:197-204 (1982); *BsuRI*: Kiss and Baldauf, *Gene* 21:111-119, (1983); and *MspI*: Walder et al., *J. Biol. Chem.* 258:1235-1241, (1983)).

3) Sub-cloning of natural plasmids

20

Another cloning approach involves transferring systems initially characterized as plasmid-borne into *E. coli* cloning plasmids (*EcoRV*: Bougueret et al., *Nucl. Acid. Res.* 12: 3659-3676, (1984); *PaeR7I*: Gingeras and Brooks, *Proc. Natl. Acad. Sci. USA* 80:402-406, (1983); Theriault and Roy, *Gene* 19:355-359 (1982); *PvuII*: Blumenthal et al., *J. Bacteriol.* 164:501-509, (1985)).

4) Multi-step cloning

Sometimes the straight-forward methylase selection method fails to yield a methylase (and/or endonuclease) clone due to various obstacles. See, e.g., Lunnen, et al., *Gene*, 74(1):25-32 (1988). One potential obstacle to cloning restriction-modification genes lies in trying to introduce the endonuclease gene into a host not already protected by modification. If the methylase gene and endonuclease gene are introduced together as a single clone, the methylase must protectively modify the host DNA before the endonuclease has the opportunity to cleave it. On occasion, therefore, it might only be possible to clone the genes sequentially, methylase first then endonuclease (see, U.S. Pat. No. 5,320,957).

5) Selection based on induction of the DNA-damage-inducible SOS response

Another method for cloning methylase and endonuclease genes is based on a colorimetric assay for DNA damage (see, U.S. Pat. No. 5,492,823). When screening for a methylase, the plasmid library is transformed into a sensitive host *E. coli* strain such as AP1-200. The expression of a methylase will induce the SOS response in an *E. coli* strain which is *McrA*<sup>+</sup>, *McrBC*<sup>+</sup>, or *Mrr*<sup>+</sup>. The AP1-200 strain is temperature sensitive for the *Mcr* and *Mrr* systems and includes a *lacZ* gene fused to the damage inducible *dinD* locus

of *E. coli*. The detection of recombinant plasmids encoding a methylase or endonuclease gene is based on induction at the restrictive temperature of the *lacZ* gene. Transformants encoding methylase genes are detected on LB agar plates containing X-gal as blue colonies. (Piekarowicz, et.al., *Nucleic Acids Res.* 19:1831-1835, (1991) and Piekarowicz, et.al. *J. Bacteriology* 173:150-155 (1991)). Likewise, the *E. coli* strain ER1992 contains a dinD1-Lac Z fusion but is lacking the methylation dependent restriction systems McrA, McrBC and Mrr. In this system (called the "endo-blue" method), the endonuclease gene can be detected in the absence of its cognate methylase when the endonuclease damages the host cell DNA, inducing the SOS response. The SOS-induced cells form deep blue colonies on LB agar plates supplemented with X-gal. (Fomenkov, et.al. *Nucleic Acids Res.* 22:2399-2403 (1994) and U.S. Pat. No. 5,498,535).

6) N-terminal-sequence-based degenerate inverse PCR method

It may occur that a modification methyltransferase gene cannot be identified (see, U.S. Pat. No. 5,945,288), or that a methylase gene can be identified but the open reading frame specifying the restriction endonuclease is uncertain. In these cases, an additional procedure for identifying the gene for the endonuclease specifically can be applied when the restriction endonuclease can be purified in sufficient quantity and purity from the original organism. In this method, the restriction

endonuclease is purified to substantial homogeneity and subjected to polypeptide sequencing. The polypeptide sequence obtained is reverse-translated into DNA sequence and degenerate PCR primers can be designed to amplify a portion of the endonuclease gene from genomic DNA of the original organism or from a gene library made therefrom. The DNA sequence of the complete genes can be obtained by methods dependent on Southern blot analysis or by further direct or inverse PCR methods. If the cognate methyltransferase gene cannot be obtained or cannot be expressed, the stability and utility of the solo restriction endonuclease clone will usually be severely compromised.

It may occur that genes for both the methyltransferase and the restriction endonuclease of a particular system can be obtained by the methods described above, but nevertheless establishment of a usable strain for enzyme production is problematic. Frequently the difficulty is with expression of the methyltransferase gene at a suitable level.

This is particularly true with method (6). Such clones sometimes can be stabilized by using heterospecific methyltransferase genes, which were not associated with the endonuclease gene in the original host but which recognize the same or a related sequence and prevent the endonuclease from cleaving its recognition sequence (see, U.S. Pat. No. 6,048,731).

It may occur that there is no suitable heterospecific methyltransferase available, and the degree of protection conferred on the host by the cognate methyltransferase is inadequate; or it may occur that apparently adequate levels of methyltransferase can be obtained but such level is toxic to the cell, resulting in strains that cannot be stored; or it may occur that protection is apparently adequate and the protected strain is viable, but the combination of the methyltransferase and the endonuclease genes gives a strain that does not express detectable endonuclease; or it may occur that protection is apparently adequate, but the combination of the methyltransferase and the endonuclease genes gives a strain that expresses detectable endonuclease, but is not sufficiently stable to make commercially useful levels of enzyme.

Many factors can be imagined that might alter the requisite level of enzyme needed for effective protection of the host cell from cleavage by a restriction endonuclease. Such factors include rapid growth, during which more DNA copies are present in the cell than are present during the stationary phase of growth; recovery from a resting state, during which time new synthesis of the modification methyltransferase may be required before new synthesis of the restriction endonuclease begins; starvation of various sorts, during which time levels of required DNA methyltransferase cofactors such as S-adenosylmethionine may be altered; and special physiological states, such as DNA

damage or other physiological insults. In addition, levels of methyltransferase can potentially be too high and become toxic, for example by binding to or methylating extraneous sites related to the cognate site and thus interfering with the reading of the DNA sequence by regulatory or DNA-condensing proteins. Thus, the absolute level of expression of the methyltransferase may need to fluctuate in response to conditions over the life of a culture, in order to be indefinitely perpetuated.

This need for a fine level of control is not unique to modification methyltransferases. Over the course of 50 years of study, many detailed regulatory schemes have been described for various sorts of functions, such as catabolic and anabolic gene sets that break down nutrients (lac, ara, gal) or synthesize essential compounds (trp, his), or response to stressors (the DNA damage response, the heat shock response). These regulatory effects are mediated by changes in promoter activity (by activators or repressors), in transcript stability (by retroregulatory elements), by alteration of translation levels (by attenuation or translational coupling), for example. Despite this high level of understanding, it is not straightforward to anticipate in advance how the demand for a function will change with physiological changes and how to achieve the desired level of a function.

Because purified restriction endonucleases, and to a lesser extent, modification methylases, are useful tools for

characterizing genes in the laboratory, there is a commercial incentive to obtain bacterial strains through recombinant DNA techniques that synthesize these enzymes in abundance. Such strains would be useful because they would simplify the task of purification as well as providing the means for production in commercially useful amounts.

**SUMMARY OF THE INVENTION**

- 10           In one embodiment, the present invention relates to a method for cloning and expressing a target restriction modification system, comprising first implementing a method for producing a balanced level of activity of a protective modification methyltransferase, such that expression compensates for changes in the physiological state of the cell and therefore confers full protection preferably during all growth phases from cleavage by the cognate restriction endonuclease; and then introducing the restriction endonuclease gene and providing for its expression.
- 20           The invention further relates to the method for producing a balanced level of activity of a protective modification methyltransferase comprising specifically testing for the extent of protection during critical growth phases which may be selected from stationary phase, the logarithmic phase of growth, recovery from storage or other growth phases, and then identifying a suitable expression vector by selecting for its function at those critical growth phases.
- 25

The above method is exemplified by the cloning and expression of the *MseI* restriction modification system, which is encoded on a DNA (deoxyribonucleic acid) fragment, which 5 fragment codes for two related enzymes, namely an enzyme which recognizes the DNA sequence 5'-TTAA-3' and cleaves the phosphodiester bond between the T residues of this recognition sequence to produce a 2 base 5' extension (Morgan R.D., *Nucl. Acids Res.*, 16:3104 (1988)) (hereinafter referred to as the *MseI* restriction endonuclease), and a 10 second enzyme, known as *M.MseI*, that recognizes the same DNA sequence, 5'-TTAA-3', but modifies this sequence by the addition of a methyl group to prevent cleavage by the *MseI* endonuclease. In addition, the invention relates to two 15 additional DNA fragments, each of which encodes an enzyme differing in sequence from *M.MseI* that perform the same function as *M.MseI*, namely modifying the sequence 5'-TTAA-3' by the addition of a methyl group thus preventing cleavage by the *MseI* endonuclease. The present invention also relates to 20 a process for preparing the DNA fragment, a vector containing the DNA fragment, a transformed host containing this DNA fragment, and an improved process for producing *MseI* restriction endonuclease from such a transformed host.

25           *MseI* restriction endonuclease produced according to the present invention is substantially pure and free of the contaminants commonly found in restriction endonuclease preparations made by conventional techniques.

The *MseI* methylase gene, but not the *MseI* endonuclease gene, was obtained generally in accordance with the technique referred to as methylase selection (U.S. Pat. No. 5,200,333, the disclosure of which is hereby incorporated by reference herein). However none of the clones obtained by methylase selection expressed detectable *MseI* restriction endonuclease activity and none was fully protected from *MseI* digestion after overnight incubation. A methylase clone was sequenced and the *MseI* methylase gene was identified based on homology to other N6-adenine methylases. Although the methylase clone did not produce any detectable *MseI* endonuclease activity, it was speculated that the endonuclease gene was likely located adjacent to the methylase gene. DNA contiguous to the *MseI* methylase gene was therefore amplified from *Micrococcus* species by inverse PCR techniques and sequenced.

To locate and positively identify the *MseI* endonuclease gene, the N-terminal amino acid sequence of highly purified *MseI* restriction endonuclease protein obtained from *Micrococcus* species was determined. An open reading frame in which the deduced amino acid sequence matched the N-terminal amino acid sequence of the *MseI* endonuclease was observed in the DNA sequence obtained by inverse PCR techniques and located 3' of the methylase gene. The *MseI* methylase gene was amplified and cloned into a vector compatible with a standard high expression vector. The *MseI*

endonuclease gene was then amplified, ligated to an expression vector such as the pET series of vectors, and introduced into a host which was pre-modified with the *MseI* methylase carried on a separate compatible vector; however, 5 no *MseI* activity was found in the few such constructs obtained. From further results below, it appears that this failure of *MseI* expression from inadequate expression of the methylase so that successful endonuclease expression became a lethal event. After obtaining a fully modifying vector in accordance with the present invention, the expression of 10 the endonuclease was also carefully regulated by construction of a vector which suppressed expression of the endonuclease during cell growth prior to the induction of the endonuclease gene. A host carrying the endonuclease and methylase genes in these special constructs was then grown, induced and harvested and used to make the *MseI* 15 endonuclease.

The preferred method for cloning and expressing the 20 *MseI* restriction-modification system consists of obtaining methylase positive clones according to methylase selection method and determining the DNA sequence of these *MseI* methylase positive clones. The DNA adjacent to the methylase gene is obtained by inverse PCR techniques and sequenced. 25 The *MseI* endonuclease protein from *Micrococcus species* is purified to near homogeneity and the N-terminal amino acid sequence determined. The *MseI* endonuclease gene is identified based on the DNA sequence and amino acid

sequence data. The expression of the *MseI* methylase is modulated to achieve full protection of the host genome without creating so much methylase expression as to be toxic to the host. This full methylation state is monitored by testing  
5 DNA obtained from cells in rapid logarithmic growth for protection from *MseI* endonuclease cleavage and using a construct which provides full protection under these rapid growth conditions. The *MseI* endonuclease is then expressed by amplifying the complete gene from *Micrococcus* species  
10 genomic DNA and ligating it into an expression vector designed to limit expression of the *MseI* endonuclease during cell growth prior to induction, such as pVR-24 (New England Biolabs, Inc., Beverly, Mass.). The construct is introduced into a host with appropriate genetic composition to provide  
15 sufficient regulatory capacity (U.S. Application Serial No. \_\_\_\_\_) which is premodified at *MseI* sites by virtue of carrying the *MseI* methylase gene expressed on the separate compatible plasmid providing full protection against *MseI* cleavage. The *MseI* endonuclease is produced by growing the  
20 host containing the *MseI* endonuclease and methylase genes, inducing with the appropriate expression conditions, harvesting the cells and purifying the *MseI* endonuclease therefrom.

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**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1A shows a restriction map of the recombinant plasmid pVR-18 encoding *MseI* DNA methyltransferase gene.

Figure 1B shows the agarose gel analysis of the susceptibility to *MseI* of pVR-18 plasmid encoding M. *MseI*. Lane 1, uncut pVR-18; lane 2, pVR-18 following overnight incubation with ten units of *MseI*; lane 3, uncut pBR322; lane 4, pBR322 following overnight incubation with ten units of *MseI*; lane 4, pVR-18 + pBR322 following overnight incubation with ten units of *MseI*; lane 5, molecular weight standard (1 kb DNA Ladder, New England Biolabs, Inc.).

Figure 2A shows a restriction map of the recombinant plasmid pEsaDix4I encoding putative DNA methyltransferase gene.

Figure 2B shows the agarose gel analysis of the susceptibility to *MseI* of pEsaDix4I plasmid encoding a putative DNA methyltransferase gene. Lane 1, uncut pEsaDix4I; lane 2, pEsaDix4I following overnight incubation with ten units of *MseI*; lane 3, uncut pUC19; lane 4, pUC19 following overnight incubation with ten units of *MseI*; lane 4, pEsaDix4I + pUC19 following overnight incubation with ten units of *MseI*; lane 5, molecular weight standard (1 kb DNA Ladder, New England Biolabs, Inc.).

Figure 3A shows a restriction map of the recombinant plasmid pEsaDix5I encoding putative DNA methyltransferase gene.

Figure 3B shows the agarose gel analysis of the susceptibility to *MseI* of pEsaDix5I plasmid encoding putative DNA methyltransferase gene. Lane 1, uncut pEsaDix5I; lane 2, pEsaDix5I following overnight incubation with ten units of *MseI*; lane 3, uncut pUC19; lane 4, pUC19 following overnight incubation with ten units of *MseI*; lane 4, pEsaDix5I + pUC19 following overnight incubation with ten units of *MseI*; lane 5, molecular weight standard (1 kb DNA Ladder, New England Biolabs, Inc.).

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Figure 4 shows the DNA sequence of *mseIM* gene (SEQ ID NO: 1) and its encoded amino acid sequence (SEQ ID NO: 2).

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Figure 5 shows the DNA sequence of *esaDix4IM* gene (SEQ ID NO:3) and its encoded amino acid sequence (SEQ ID NO:4).

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Figure 6 shows the DNA sequence of *esaDix5IM* gene (SEQ ID NO:5) and its encoded amino acid sequence (SEQ ID NO:6).

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Figure 7 shows the DNA sequence of *mseIR* gene (SEQ ID NO:7) and its encoded amino acid sequence (SEQ ID NO:8).

Figure 8 shows a restriction map of the recombinant plasmid pNKR1707*mseIM* used for construction of a library of

constitutive promoters randoming mutagenized by error-prone PCR.

Figure 9A shows a restriction map of the recombinant plasmid pNKR1707mseIM-9 encoding the *MseI* DNA methyltransferase gene and upstream regulatory elements.

Figure 9B shows the DNA sequence upstream of *MseI* DNA methyltransferase gene (SEQ ID NO:9) which contains an optimal promoter sequence.

Figure 10 shows the construction of the plasmids pVR-26 and pVR-27 used for controlled expression of *MseI* DNA methyltransferase gene.

Figure 11 shows the construction of the pVR-24 expression vector.

Figure 12A shows a restriction map of pVR-25 encoding the *MseI* restriction endonuclease gene.

Figure 12B shows the mechanism of action of the tight regulatory system in pVR-25 for cloning genes encoding cytotoxic proteins.

Figure 13 shows a restriction map of pCEF-8 encoding T7 lysozyme gene.

Figure 14 shows an assay of *MseI* restriction endonuclease activity in crude cell extracts made from *E. coli* strains MseRM4, MseRM5 and MseRM6. The growth conditions are described in Example IV.

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Figure 15 shows an assay of *MseI* restriction endonuclease activity in crude cell extracts made from *E. coli* strain MseRM4 (NEB #1284) after growth in the 100-liter fermenter.

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#### **DETAILED DESCRIPTION OF THE INVENTION**

In one embodiment, the present invention relates to a method of producing a target restriction endonuclease by first providing a vector expressing a modification methyltransferase gene protecting DNA from restriction enzyme cleavage, in such a form that complete protection of the host DNA is observed preferably at all growth phases in which the cognate restriction endonuclease is present without leading to toxicity (a fully-protecting methyltransferase vector), followed by providing a vector expressing the desired restriction endonuclease gene. The present invention is not limited by the identity of the modification methyltransferase gene or restriction enzyme, except that the modification methyltransferase must protect against cleavage by the said restriction enzyme.

RECORDED IN U.S. PATENT AND TRADEMARK OFFICE

In a preferred embodiment, the fully-protecting methyltransferase vector may be obtained by identifying regulatory elements capable of driving methyltransferase expression to provide full protection during a phase of growth that is especially sensitive to methyltransferase expression pattern. In accordance with the present invention, this may be done by the following steps:

(1) obtaining a methyltransferase gene in a vector by methods known in the art;

(2) placing a regulatory element such as a promoter in a suitable location with respect to the gene;

(3) transformation into the desired host cell;

(4) reisolation of vector from the pooled transformants during the time that they are in the logarithmic phase of growth;

(5) selection by digestion with the endonuclease; and

(6) retransformation of the surviving undigested and thus protected vector population into a fresh host.

It will be understood by those skilled in the art that step (4) of this procedure may be performed with pooled vector isolated from logarithmic phase or from various other phases of growth, for example from stationary phase, from a resting state achieved by starvation for carbon or nitrogen or other essential nutrient, or from cells in a special physiological state, such as a state of DNA damage, or in the presence of

physiological insults such as acidic media or toxic compounds, as may be appropriate.

In a preferred embodiment, the regulatory element of step (2) is identified by a procedure comprising the following steps:

- (a) cloning into the vector containing the methyltransferase gene, at a desired location, a pool of fragments containing various distinct regulatory elements; and
- (b) proceeding with steps (3) through (6).

It will further be understood by those skilled in the art that the process of selection comprising steps (3) through (6) may be repeated to select further improvement.

It will further be understood by those skilled in the art that step (2a), cloning a pool of fragments containing regulatory elements at the same or a different location, may be repeated followed by repeated selection as may be appropriate.

The present invention is not limited by the identity of the regulatory element, which may be a promoter, an operator, an enhancer, or a down-stream regulatory element.

In a preferred embodiment, the methyltransferase gene of step (1) is isolated by the methylase selection procedure

(U.S. Pat. No. 5,200,333). The present invention is not limited to methyltransferase genes isolated in this way but includes genes isolated by any of the methods described above such as phage selection, subcloning of natural plasmids,

5 identification based on induction of the DNA-damage-inducible SOS response, by inverse PCR based on amino acid sequence of a purified protein, or identification in sequence databases from similarity to sequences of other methyltransferase followed by cloning by PCR or by Southern blot based  
procedures (see e.g., Kong, et al., *Nucleic Acids Res.* 28:3216-  
10 3223 (2000)).

In a preferred embodiment, the collection of distinct regulatory elements of step (2a) comprises copies of the *his* promoter of *S. typhimurium* randomly mutagenized by error-prone PCR together with such contaminating chromosomal fragments as may be present in the preparation of mutagenized fragments. The present invention is not limited to fragments obtained in this way, but may include collections 15 of fragments isolated from genomic DNA of *E. coli* or another organism or fragments derived by oligonucleotide synthesis with degenerate sequences at random or specific locations or fragments derived by recombinational PCR of a random or specific collection of fragments. In a preferred embodiment, 20 the regulatory element obtained in this way is the sequence of SEQ ID NO:9.  
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USPTO Patent Application  
Sequence Listing  
Sequence 1

It will further be understood by one skilled in the art that this method may be applied to any methyltransferase that confers protection from cleavage by the restriction endonuclease in question, not merely that which co-occurs with the said endonuclease in a particular natural isolate.

The present invention further relates to the isolation of methyltransferase genes of desired specificity from DNA of environmental sources without first culturing the organisms contained therein. In a preferred embodiment, these genes are isolated by methylase selection from DNA made from a sample of a mixed green filament and mat community of prokaryotes growing at 68°C at Dixie Valley Hot Spring, Nevada.

The present invention further relates to provision of a desired restriction endonuclease gene expressed from a vector with tight regulation such that extremely low levels of protein are expressed in the absence of induction (very low basal expression is observed). In a preferred embodiment this tight regulation vector comprises a vector with antagonistic and independently regulatable promoters reading through the cloned target gene as described in WO 99/11821 and U.S. Application Serial No. 09/486,356 but basal expression has been further lowered by providing for a lower copy number than is present in the previously existing vector pLT7K used for this purpose. In a preferred embodiment, the copy number of the vector is lowered by

exchanging the replication origin of pLT7K for that of pACYC184. Other replication origins might also be used, such as those of pSC101 (Stoker, et al., *Gene* 18:335-341 (1982)), pSYX20 (U.S. Patent No. 5,262,313), F (Shizuya, et al., *Proc. Natl. Acad. Sci. USA* 89(18):8794-8797 (1992)) or other low-copy vectors (Harayama, et al., *Mol. Gen. Genet.* 184:52-55 (1981) and Wohlfarth, et al., *J. Gen. Microbiol.* 134:433-440 (1988)). In a preferred embodiment, the vector is pVR-24.

In a preferred embodiment, further lowering of basal expression level is achieved by employment of a strain expressing high levels of the negative regulator of expression in the direction that allows translation of the target gene, as described in the accompanying U.S. Application Serial No.

The above described method is exemplified in another embodiment of the present invention, namely the cloning and expression of the *MseI* restriction-modification system.

The present invention also provides novel DNA constructs and novel compositions comprising microbial strains producing *MseI* restriction endonuclease. The restriction endonuclease of interest in the present invention, *MseI*, recognizes the DNA sequence 5'-TTAA-3' and cleaves the phosphodiester bond on between the T residues of this recognition sequence to produce a 2 base 5' extension.

In order to overexpress the *MseI* restriction endonuclease, additional steps beyond the well-known art of the methylase selection procedure (U.S. Pat. No. 5,200,333) are required, including particularly the fine balance of *MseI* methyltransferase expression to fully protect the host genomic DNA from *MseI* digestion *in vivo* while yet not producing so much methyltransferase as to be toxic to the host. A vector, containing the *mseIM* gene optimized for expression such that full protection against *MseI* endonuclease is observed even during very rapid (logarithmic stage of) cell growth, is first used to modify an *E. coli* host. This host is then transformed with a compatible vector, such as pVR-25, containing the *mseIR* gene followed by selection for colonies that contain both vectors on the appropriate antibiotic plates. *MseI* endonuclease producing constructs are identified by growing individual transformants and assaying for *MseI* endonuclease activity, (as in Example V below).

The method described herein by which the *MseI* methylase gene and the *MseI* restriction endonuclease genes are preferably cloned and expressed in *E. coli* employs the following steps:

1) Cloning of the DNA methyltransferase genes which protect from *MseI* cleavage.

It is well known that DNA modification methylases recognize and bind to the same nucleotide recognition

sequence as the corresponding restriction endonuclease, but instead of breaking the DNA, they chemically modify one or other of the nucleotides within the sequence by the addition of a methyl group. Following this methylation, the recognition sequence is no longer bound or cleaved by the restriction endonuclease. The DNA of a bacterial cell is always fully modified, by virtue of its modification methylase, and it is therefore completely insensitive to the presence of the endogenous restriction endonuclease. In this situation, only unmodified, and therefore identifiably foreign, DNA that is sensitive to restriction endonuclease recognition and attack.

The first step of present method is to identify the DNA methyltransferase gene which protects from *MseI* cleavage. To accomplish this the DNA methylase from *Micrococcus species* (NEB446) can be cloned. Alternatively, a DNA methyltransferase from an R-M system other than the *MseI* R-M system, but able to protectively modify DNA to prevent digestion by the *MseI* restriction enzyme can be identified as described in U.S. Pat. No. 5,179,015. In the present invention, three DNA methylases able to protect DNA from digestion by the *MseI* restriction enzyme were identified.

First, the total genomic DNA was purified from *Micrococcus species* (NEB#446). A random library of this DNA was constructed by partially digesting the DNA with a frequent cutting endonuclease, *Sau3AI*, to produce fragments of approximately 1 to 10 kilobases (kb) in length. These fragments were ligated into a vector pBR322, previously

cleaved with *Bam*HI and dephosphorylated. The ligation reaction was transformed into chemically competent *E. coli* ER2502 cells. The transformants were pooled, and the plasmid was population purified to form the primary plasmid library. An aliquot of these purified plasmids was digested with *Mse*I restriction endonuclease to destroy all plasmids which had not expressed the *Mse*I methylase gene *in vivo* and thus protected the plasmid DNA from digestion. The digested plasmid pool was transformed again into *E. coli* ER2502 to recover the intact, *Mse*I methylase expressing plasmids. Individual clones were picked, there plasmid DNA was purified and challenged by cleavage with *Mse*I endonuclease. Plasmids which were not cut by *Mse*I contained the *Mse*I methyl-transferase gene.

In a preferred embodiment, the methyltransferase gene is one protecting against the restriction endonuclease *Mse*I obtainable from *Micrococcus species* (NEB#446), and may be selected from among the set of sequences that can encode proteins specified in SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:7. These proteins may be encoded for example by those DNA sequences set forth in SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:6.

To search for alternative DNA methyltransferases that are able to protect DNA from cleavage by *Mse*I endonuclease, a library of clones from a source of DNA other than *Micrococcus* species may be constructed in a vector containing one or

more *MseI* restriction sites. This library of clones is then selected by one or more rounds of *MseI* digestion to destroy non-protecting clones followed by transformation of the digested plasmids to recover protected clones. Such a library was created from DNA (designated "environmental DNA") isolated from a sample of a mixed green filament and mat community of prokaryotes growing at 68°C at Dixie Valley Hot Spring, Nevada. Purified environmental DNA was digested with *NsiI* endonuclease and ligated into the vector pNEB193 previously cleaved with *PstI* restriction endonuclease and dephosphorylated. The ligation reaction was transformed into *E. coli* ER2683 by electroporation. The transformants were pooled and the plasmid population was purified to form the primary plasmid library. An aliquot of these purified plasmids was digested to completion with an excess of *MseI* restriction endonuclease and used to transform ER2683. Plasmids of the resulting transformants were miniprepped and analyzed by *MseI* restriction enzyme digestion and subsequent agarose gel electrophoresis. 9 plasmids examined were found to be resistant to *MseI* digestion and each was found to encode one of two different methylase genes that each function to protect DNA from cleavage by *MseI*. These two methylases were named *esaDix4IM* (SEQ ID NO:3 and SEQ ID NO:4) and *esaDix5IM* (SEQ ID NO:5 and SEQ ID NO:6). Analysis of crude cell extracts prepared from these clones revealed no endonuclease activity. These methyltransferases, or others like them, may be used to protect a host's own DNA and thus enable the successful expression of the *MseI* endonuclease.

2) Sequence determination of the entire *MseI* restriction-modification system.

5           The *MseI* methylase gene, but not the *MseI* endonuclease gene, was obtained generally in accordance with the technique referred to as methylase selection (U.S. Pat. No. 5,200,333) as above in step 1. However none of the clones obtained by methylase selection expressed detectable  
10           *MseI* restriction endonuclease activity. A methylase clone was sequenced using standard techniques on an ABI 373 DNA sequencing machine. The *MseI* methylase gene was identified based on amino acid homology to other N6-adenine methylases. Although the methylase clone did not produce any detectable *MseI* endonuclease activity, it was speculated  
15           that the endonuclease gene was likely located adjacent to the methylase gene. DNA contiguous to the *MseI* methylase gene obtained from *Micrococcus* species (NEB#446) was therefore amplified from *Micrococcus* species genomic DNA by inverse PCR techniques and sequenced.  
20

25           To locate and positively identify the *MseI* endonuclease gene, the N-terminal amino acid sequence of highly purified *MseI* restriction endonuclease protein obtained from *Micrococcus* species was determined. *MseI* endonuclease may be purified from *Micrococcus* species (NEB#446) as set forth in Example III below. An open reading frame in which the deduced amino acid sequence matched the N-terminal amino

acid sequence of the *MseI* endonuclease was observed in the DNA sequence obtained by inverse PCR techniques which was located 3' of the methylase gene.

5           Alternatively, the N-terminal amino acid sequence of *MseI* restriction endonuclease can be used to design degenerate oligonucleotide primers for PCR amplification of a portion of the *MseI* endonuclease gene from *Micrococcus* species (NEB#446). The DNA sequence obtained can then be used to guide inverse PCR amplification of the DNA on either side of this original portion of the *MseI* endonuclease gene, and the *mseIM* and *MseI* genes can be identified in this DNA sequence as above. Both methods were used for cloning and sequence determination of the entire *MseI* restriction-modification system.

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3) Fine optimization of the *MseI* methyltransferase expression

20           Once the complete genes for the *MseI* endonuclease and *MseI* methyl-transferase have been identified (SEQ ID NO:7, SEQ ID NO:8 and SEQ. ID. NO:1 and SEQ ID NO:2, respectively), they may then be manipulated in a variety of ways to provide for expression. Using the methylase constructs obtained as above, expression of the *MseI* restriction endonuclease gene under T7 promoter control using the pET series of vectors (Novagen Inc., Madison, WI)

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was vigorously attempted but failed to yield a *MseI* restriction endonuclease producing clone.

A unique combination of methods, including the introduction of a second, controllable promoter before the methylase gene, using a low copy replicon for the endonuclease gene and increasing the copy number of LacI repressor in the host prior to the introduction of the endonuclease gene, was used to control the overexpression of recombinant *MseI* endonuclease.

It was observed that the methylase constructs obtained by methylase selection did not fully protect the host *E. coli* chromosomal DNA when the cells were rapidly growing in logarithmic phase of growth. In order to increase expression of the methylase, and thus fully protect the host DNA so that *mseIR* could be introduced successfully into the cells and expressed, the methylase gene was amplified from *Micrococcus species* DNA and cloned into a family of vectors (pNK series, see Example IV below) under the expression of various strength constitutive promoters. In this attempt, no methylase constructs were obtained for the two highest level of expression promoters, due we believe to toxicity to the cell from too much expression of the methylase. Constructs with the two lower level of expression promoters failed to fully protect the host against *MseI* cleavage when checked at logarithmic phase of growth. In order to increase methylase expression to fully protect the host DNA during rapid growth

but remain below the level of toxicity, one of the promoter constructs was subjected to random mutagenesis by error-prone PCR in the promoter region. Mutated clones expressing *MseI* methylase were selected using the methylase selection technique referenced above, and then individual clones were tested for the ability to fully protect host genomic DNA from *MseI* cleavage during rapid logarithmic growth by harvesting cells during logarithmic growth, purifying DNA from these host cells and testing for full protection from *MseI* cleavage. One of the constructs found to fully protect against *MseI* was then used for the expression of the *MseI* endonuclease.

This method of modulating expression of a methyltransferase to achieve full protection during all stages of host cell growth may prove applicable to other systems where the endonuclease proves difficult to express, or express instability in a host cell (see, U.S. Patent No. 6,025,179 and 6,048,731).

4) Expression of the *MseI* restriction endonuclease under the control of an inducible promoter

To optimize expression of recombinant *MseI* of the present invention, inducible or constitutive promoters are well known and may be used to express high levels of an *mseIR* gene in a recombinant host. Similarly, high copy number vectors, well known in the art, may be used to achieve high levels of expression. In accordance with the present

invention, it has been found that a particularly preferred method for expression of *MseI* restriction endonuclease is an expression vector designed to limit expression of the *MseI* endonuclease during cell growth prior to induction, such as  
5 pVR-24 (New England Biolabs, Inc., Beverly, Mass.). This plasmid contains the segment encoding replicative function (ori), a chloramphenicol-resistance gene (Cm), gene encoding kanamycin resistance which is flanked by restriction endonuclease sites suitable for cloning. The *cI857* gene encodes a mutant form of the lambda bacteriophage repressor protein, which conditionally binds to DNA sequences (the CI operator) that overlap PL and PR (the lambda bacteriophage major leftward and rightward promoters, respectively). The *lacI* gene encodes a repressor protein, LacI, that conditionally binds a DNA sequence (the lac operator) which has been constructed to overlap  $P_{T7}$  (bacteriophage T7 RNA polymerase transcriptional promoter). Briefly, at high temperature (42°C) without IPTG, the antisense promoter is active, while  $P_{T7}$  is repressed by LacI. At 30°C and with IPTG expression occurs from  $P_{T7}$  (see Figures 11 and 12). At intermediate temperatures and with intermediate IPTG concentrations, intermediate levels of expression can be obtained.

25 To obtain a stable clone which overexpresses the restriction endonuclease, the host is generally pre-protected from restriction endonuclease digestion. In the present invention this is accomplished by cloning the *MseI* methylase

gene, or another methylase gene that protects against *MseI* cleavage, such as *esaDix4IM* or *esaDix5IM*, expressed on the separate compatible plasmid in a manner providing full protection against *MseI* cleavage. As shown in the Example V below it was found that the stability of the expression 5 plasmid containing the restriction endonuclease gene construct and/or its mRNA could be improved when the *MseI* methyltransferase gene is preceded by a DNA fragment encoding a novel promoter sequence. The *MseI* endonuclease is produced by growing the host containing the *MseI* endonuclease and the protective methylase gene, inducing 10 with the appropriate expression conditions, harvesting the cells and purifying the *MseI* endonuclease therefrom.

The invention further provides a process for producing 15 the *MseI* restriction endonuclease, in which recombinant DNA modification methods are used for transforming a microorganism such that the gene encoding the *MseI* restriction endonuclease and a gene coding for a DNA 20 methyltransferase which protects the host DNA from *MseI* cleavage are introduced into said microorganism, the organism is grown under conditions suitable for expression of *MseI* endonuclease, harvested and the *MseI* endonuclease is purified therefrom.

25 Although the above-outlined steps represent the preferred mode for practicing the present invention, it will be apparent to those skilled in the art that the above described

approach can vary in accordance with techniques known in the art.

5           The following Examples are given to illustrate embodiments of the present invention as it is presently preferred to practice. It will be understood that these Examples are illustrative, and that the invention is not to be considered as restricted thereto except as indicated in the appended claims.

10           The references cited above and below are herein incorporated by reference.

#### **EXAMPLE I**

##### **Cloning of the *MseI* methyltransferase gene (*mseIM*).**

15           *Micrococcus* species (NEB#446) was grown overnight in 1L of LB broth, the cells were harvested and genomic DNA was isolated using Qiagen Genomic-tip 100/G Genomic DNA Purification Kit (Cat. No. 10243) according to the manufacturer's instructions. Genomic DNA was partially digested with *Sau3AI* to produce fragments from 1 to 10 kb, and 20 ug of this cleaved genomic DNA was ligated with 3 ug of *BamHI*-digested and dephosphorylated pBR322. The ligation mixture was transformed into *E. coli* strain ER2502. Approximately 100,000 transformants were obtained. The transformants were pooled, grown in 500 ml LB broth containing 100 $\mu$ g/ml ampicillin, and the plasmid population

was purified to form the primary plasmid library. 2 micrograms of this plasmid library was digested to completion with an excess of *MseI* restriction endonuclease and used to transform ER2505. Plasmids of the resulting transformants were subjected to a second round of selection. 80 transformants were obtained and the plasmid DNA of 16 of these was analyzed by *MseI* restriction enzyme digestion and subsequent agarose gel electrophoresis. 14 out of 16 plasmids examined were found to be resistant to *MseI* digestion and found to carry the same *mseIM* gene (SEQ ID NO:1, SEQ ID NO:2) on a *Sau3AI* fragment of approximately 1.6 kb. Analysis of crude cell extracts prepared from those 14 clones revealed no *MseI* activity.

**EXAMPLE II**

**Cloning two DNA methylases from an environmental DNA sample that protect DNA from cleavage by *MseI* endonuclease .**

To search for alternative DNA methyltransferases that are able to protect DNA from cleavage by *MseI* endonuclease, a library of clones from a source of DNA other than *Micrococcus* species (NEB446) may be constructed in a vector containing one or more *MseI* restriction sites. This library of clones is then selected as above by one or more rounds of *MseI* digestion to destroy non-protecting clones followed by transformation of the digested plasmids to recover protected clones, as in Example I above. Such a library was created from DNA isolated from a sample of a mixed green filament

and mat community of prokaryotes growing at 68°C at Dixie Valley Hot Spring, Nevada. 2 micrograms of the DNA was digested with *Nsi*I endonuclease and ligated into 1 microgram of the vector pNEB193 previously cleaved with *Pst*I and dephosphorylated. The ligation reaction was transformed into *E. coli* ER2683 by electroporation and approximately 1,000,000 transformants were obtained. The transformants were pooled, grown in 500 ml LB broth containing 100 $\mu$ g/ml ampicillin, and the plasmid population was purified to form the primary plasmid library. 1 microgram of this plasmid library was digested to completion with an excess of *Mse*I restriction endonuclease and used to transform ER2683. Plasmids of the resulting transformants were miniprepped and analyzed by *Mse*I restriction enzyme digestion and subsequent agarose gel electrophoresis. 9 plasmids examined were found to be resistant to *Mse*I digestion and were found to encode one of either two different methylase genes that both function to protect DNA from cleavage by *Mse*I. These two methylases were named esaDix4IM and esaDix5IM (SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5 and SEQ ID NO:6). Analysis of crude cell extracts prepared from these clones revealed no endonuclease activity. These methyltransferases, or others like them, may be used to protect a host's own DNA and thus enable the successful expression of the *Mse*I endonuclease.

### EXAMPLE III

**Identification and sequence determination of the *MseI* restriction endonuclease gene using N-terminal amino acid sequence and DNA sequence adjacent to the *MseI* methylase obtained by the inverse PCR method.**

A) Purification of the *MseI* restriction endonuclease from *Micrococcus species* to near homogeneity:

10       *Micrococcus species* (NEB#446) cells were propagated in LB media at 30°C. The cells were harvested by centrifugation after 20 hours growth and stored at -70°C until used. All of the procedures were performed on ice or at 4°C. The *MseI* endonuclease was purified following the same scheme as in Example VI. Approximately 10,000 units of *MseI* activity were purified to near homogeneity. 16 µl of the peak fraction was loaded onto an SDS-PAGE protein gel and subjected to electrophoresis. The gel was stained with Coomassie blue R-250 and a prominent band at approximately 21 kD corresponding to the *MseI* restriction endonuclease activity was observed.

B) Amino Terminal *MseI* protein sequence:

25       The *MseI* restriction endonuclease, prepared as described, was subjected to electrophoresis and electroblotted according to the procedure of Matsudaira (Matsudaira, P., *J. Biol. Chem.* 262:10035-10038 (1987), with modifications as previously described (Looney, et al., *Gene* 80:193-208 (1989)). The membrane was stained with

Coomassie Blue R-250 and the protein band of approximately 21 kd was excised and subjected to sequential degradation on an Applied BioSystems Division, Perkin-Elmer Corporation (Foster City, California) Model 407A gas phase protein sequencer (Waite-Rees, et al., *J. Bacteriol.* 173:5207-5219 (1991)). The first 25 residues of the 21 kD protein corresponded to (Met)-Thr-His-Glu-Pro-Thr-Asp-Asp-Pro-Asp-Phe-Ile-Val-Met-Ala-Ala-Ser-Ala-Xxx-Asn-Leu-Ala-Asp-Xxx-Tyr (SEQ ID NO:10). This data was used to compare with amino acid sequence deduced from the DNA sequence adjacent to the methylase gene to identify the endonuclease gene.

C) DNA sequence determination adjacent to the *mseIM* methylase:

Template preparation for inverse PCR amplification: 1 µg of *Micrococcus* species (NEB#446) DNA was digested with 10 units of *Hae*II restriction endonuclease in 1X NEBuffer #4 in a 50 µl reaction volume for 1 hour at 37°C. The *Hae*II enzyme was heat inactivated by incubating at 75°C for 20 minutes. The *Hae*II digested DNA was circularized by adding 50 µl 10X T4 DNA ligase buffer and 400 µl dH<sub>2</sub>O, followed by 5 µl (2000 NEB units) T4 DNA ligase (NEB#202) and incubating at 16°C for 16 hours. A portion of this circularization ligation reaction was then used as the template for subsequent inverse PCR reactions.

Primers MseI-IP1 and MseI-IP2 of sequences shown below were synthesized. These primers hybridize within the *MseI* endonuclease gene and are oriented in the opposite direction relative to each other.

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## Primer MseI-IP1

5'-CTTCTGCAGCCGATTCATAGTGATGGC -3' (SEQ ID NO:11)

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## Primer MseI-IP2

5'- GTTCTGCAGATCGGGATCATCCGTCGG -3' (SEQ ID NO:12)

In the reaction that was successful in amplifying the product, a reaction mix was made by combining:

15 10 µl of 10X Vent® reaction buffer

6 µl of 4 mM dNTP solution

5 µl of primer MseI-IP1 at 10 µM concentration

5 µl of primer MseI-IP2 at 10 µM concentration

3 µl of 100 mM MgSO<sub>4</sub> (5 mM Mg<sup>++</sup> final concentration)

12.5 µl of circularized DNA template (approximately 25 ng)

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58 µl dH<sub>2</sub>O

2 µl (4 units) of Vent® Exo- polymerase NEB#257

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The PCR amplification conditions were: 95°C for 3 minutes for one cycle, followed by 4 cycles of 95°C for 30 seconds, 52°C for 30 seconds and 72°C for 1.5 minutes, followed by 20 cycles of 95°C for 30 seconds, 62°C for 30

seconds and 72°C for 1.5 minutes. 10 µl of the PCR reaction was analyzed by electrophoresis on a 0.8 % agarose gel.

An approximately 1350 bp product was observed in the  
5 *Hae*II circular template PCR reaction. The product was gel purified and suspended in 25 µl DNA (1X TE) buffer. This PCR product was then sequenced on an ABI 373 automated sequencing system according to the manufacturer's instructions, using the PCR primers above as the sequencing primers. Additionally, the *Mse*I endonuclease region was PCR amplified in a like reaction with the following primers and the PCR product was sequenced.

10 Primer *Mse*I-IP3

15 5'-GGTTCTGCAGTTAACATATGACCCACGAACCGACG  
GATG-3' (SEQ ID NO:13)

20 Primer *Mse*I-IP4

5'-GTTGGATCCGTCGACGCTTCTCGGCGTACCGAGCG-3'  
(SEQ ID NO:14)

The *Mse*I endonuclease gene is identified by comparing the amino acid translation of DNA sequences adjacent to the *Mse*I methylase gene with the amino acid sequence data obtained from N-terminal amino acid sequencing of the *Mse*I endonuclease. An open reading frame oriented in the same direction as the *Mse*I methylase gene and overlapping the methylase gene by 7 amino acid residues was found in which  
25

the first 25 amino acids coded for in the DNA sequence matched the amino acid sequence determined from the *MseI* endonuclease protein.

5            Sequencing of the insert carrying the *MseI* methylase and restriction endonuclease genes was performed using GPS®-1 of the Genome Priming System (New England Biolabs, Beverly, Mass.). GPS®-1 contains a modified Tn7 with the nptII gene for resistance to kanamycin, and insertions were generated in vitro in pVR-18 and pNEB193 containing part of the *MseI* methylase gene according to the instructions of the manufacturer (New England Biolabs, Beverly, MA). These insertions were then sequenced using an ABI 373 automated sequencing system according to the manufacturer's instructions, using the primers included in GPS®-1 kit (Primer S and Primer N for the left and right end of the Transprimer, respectively)

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#### **EXAMPLE IV**

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##### **Optimization of the *MseI M* expression**

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1) Placing the *MseI* methylase gene under different strength of constitutive promoters

To achieve a range of constitutive expression of the *MseI* methylase, a related family of pNK vectors (generous gifts from N. Kleckner) containing constitutive promoters of different strengths was utilized. These plasmids contain either

the WT or mutated pHis promoters upstream of a *Bam*HI site and are derivatives of the RS415 plasmid (Simons, et al. *Gene*, 53 (1987) 85-96 ). Their designations and promoter strength are as follows:

5

No.	Plasmids	Promoter Strength
1	pNK1707 (wildtype)	1x
2	pNK2213	20x
3	pNK1786	100x
4	pNK2138	1070x

The above plasmids were digested by *Bam*HI, *Mun*I and *Ban*II and the vector backbones containing the constitutive promoters were gel purified. (The *Ban*II digest was included to aid in gel purification of the vector backbone by eliminating a similarly sized plasmid fragment.)

To prepare the *Mse*I methylase gene for insertion downstream of the constitutive promoters described above, PCR was done using Vent® DNA polymerase, 1X ThermoPol buffer, 4 mM MgSO<sub>4</sub>, 80 ng of pVR19 plasmid (R. Vaisvila) containing the *Mse*I methylase gene as the template in a 100 µl PCR reaction, and primers introducing an upstream *Bam*HI site 5'-GAACCGGATCCGACCCTGAGTGAGAACATGCC-3' (SEQ ID NO:15) and a downstream *Mfe*I site 5'-AGGTCGCAATTGCCAGG GGTCGTCTTCACTCGCTAC-3' (SEQ ID NO:16) with respect to the methylase gene. Twenty-five cycles were done consisting of 10 sec at 95°C, 60 sec at 60°C and 75 sec at 72°C. The resulting 1019 bp PCR product was purified using a QiaQuick

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PCR purification protocol, digested sequentially by *Bam*HI and *Mun*I, and purified once again using the QiaQuick PCR purification protocol.

5           The *Mse*I methylase gene was ligated into all four *Bam*HI-*Mun*I vector backbones, transformed into ER2688 cells, and plated on Luria-Bertani (supplemented with 1 gram glucose and 1 gram MgCL<sub>2</sub> per liter; subsequently referred to as supplemented LB) agar plates. However, attempts to place the *Mse*I methylase under the highest two levels of expression failed, assumingly due to instability from high levels of methylation in the cells. Constructs containing the lower two levels of expression (pNKR1707*Mse*Im, pNKR2213*Mse*Im did not result in full methylation of the cellular DNA, as judged by susceptibility of purified plasmid DNA from these cells to restriction by *Mse*I (1 µg plasmid DNA in 50 µl volume, 20 units *Mse*I, 1 hour at 37°C).

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20           2) Construction of a library of randomly-mutagenized constitutive promoters by error-prone PCR

25           To find an intermediate level promoter construct for the *Mse*I methylase between that of pNKR2213*Mse*Im and the apparently unstable pNKR1786*Mse*Im, the constitutive promoter region was subjected to random PCR mutagenesis and selection. The mutagenesis protocol employed high levels of Taq DNA polymerase (5 units/100 µl reaction volume), unequal dNTP pools (1.2 mM dCTP and TTP; 0.2 mM dATP and

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dGTP), high levels of MgCl<sub>2</sub> (7 mM), presence of MnCl<sub>2</sub> (0.5 mM), 2 ng of the pNKR1707MseIm per 100 µl volume and high PCR cycle numbers (35). The primers flanked the *MseI* methylase gene at the *AgeI* and *BamHI* restriction sites respectively 5'-GCGATACAGACCGGTTCAGACAGGATAAAG-3' (SEQ ID NO:17) and 5'-GGTCGGATCCGGCGATACAGCGAG-3' (SEQ ID NO:18).

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After PCR, the mutated promoter copies were restricted by *AgeI* and *BamHI*, gel purified with a Qiagen gel purification kit, and ligated into a *AgeI*-*BamHI* restricted pNKRMseIm construct that had been purified away from its endogenous constitutive promoter. Following electroporation into competent ER2688 cells, 20,000 colonies were achieved. These colonies were pooled and the plasmids were purified using a Qiagen purification protocol. This constituted a library of randomly mutagenized constitutive promoters, upstream of the *MseI* methylase gene.

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3) Selection of clones yielding plasmids resistant to *MseI* restriction

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To select for plasmids possessing a mutated constitutive promoter resulting in a stable, high level of methylation, 5 µg of the plasmid library was challenged by *MseI* restriction (5 µg DNA, 50 units *MseI* for 4 hrs at 37°C, followed by a 20 min incubation at 65°C to inactivate the *MseI* restriction endonuclease. A portion of the challenged pool (250 ng) was

transformed into calcium-competent ER2688 cells and plated on supplemented LB agar plates and grown overnight at 37°C. This resulted in 63 colonies.

5 Six of these 63 colonies were randomly selected for further individual examination; after overnight growth in 10 ml supplemented LB medium, plasmid DNA was purified using a Qiagen Qia-prep spin miniprep protocol. When 100 ng of the purified plasmid DNA was challenged with 20 units of *MseI* for 30 minutes at 37°C, all 6 were found to be fully restricted, indicating an inadequate level of methylation.

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15 The remaining 57 colonies were pooled and a plasmid purification was done using a Qiagen plasmid purification protocol. From this plasmid pool, 50 ng was subjected to a longer (overnight) 50 unit *MseI* challenge, followed by a 20 min incubation at 65°C to inactivate the *MseI* restriction endonuclease. A portion of the challenged pool (4 ng) was transformed into calcium-competent ER2688 cells, plated on supplemented LB agar plates and grown overnight at 37°C.  
20 This resulted in 13 colonies.

25 Nine of these 13 colonies were randomly selected for further individual examination; after overnight growth and plasmid purification as previously described, 7 of the 9 were found to be fully methylated when 1 µg plasmid DNA was incubated with 50 units *MseI* in a 50 µl reaction volume overnight at 37°C.

5

To further establish the level of methylation present in the cells, the 7 colonies were harvested for plasmid purification during the *logarithmic* phase of culture growth (cells were harvested 4 hours at 37°C after a 1:100 dilution of an overnight culture into fresh supplemented LB growth medium). Such cells would be expected to be replicating their DNA at such a rate that methylation by an expressed *MseI* methylase might be unable to achieve complete methylation. Plasmid DNA was purified from these logarithmically growing cultures using Qiagen Qia-prep purification protocols and 0.5 ug of this plasmid DNA was incubated overnight at 37°C with 50 units *MseI*. Using this more difficult methylation standard, 3 of the 7 colonies were fully protected (methylated) and resistant to restriction.

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The three clones (#4, #9 and #10) resulting in a stable and full level of *MseI* methylation had their promoter regions examined by mapping with *AgeI* and *BamHI*, and sequencing using a primer with an annealing position upstream of the promoter region. (5'-GGATCTTCCAGTGGTGCATGAACG-3' (SEQ ID NO:19). Two of the 3 clones (#9 and #10) were identical; thus the two step selection process described resulted in finding two independent promoters that yield a stable, full level of *MseI* methylation.

Unexpectedly both promoter #4 and promoter #9/#10 were not mutagenized constitutive promoters as had been

the experimental design, but instead were *AgeI-BamHI E. coli* sequences that must have originated from the low level of *E. coli* DNA contamination present in the plasmid preparations.

5           The #4 promoter, by *AgeI/BamHI*, mapping appeared to be approximately 1000 bp in length; by sequencing, the first 438 bp were identical to *E. coli* K-12 MG1655 section 349 (Accession No. AE000459), base # 7813—8251. Upon examination of the sequence data, a *BamHI* site was found at base #8814, which would yield the *AgeI-BamHI E. coli* fragment of 1002 bp. This *E. coli* sequence contains the 5' end of the *yigW\_2* orf and two predicted promoters, one of which is oriented in the same direction as the *MseI* methylase (#8672-8704).

10           The #9/#10 promoter mapping appeared, by *AgeI/BamHI*, to be approximately 420 bp in length; by sequencing, the promoter was identical to *E. coli* K-12 MG1655 section 41 (Accession No. AE000151), base # 2511-2998. This defines a 488 bp *AgeI-BamHI E. coli* fragment that contains the 5' end of the *cof* orf and two predicted promoters oriented in the same direction as the *MseI* methylase at positions #2605-2632 and #2714-2742. This #9/#10 sequence was used for further work.

4) Further optimization of *MseI* methylase expression

Using the strategy described above, a level of *MseI* methyltransferase expression which allowed expression of the *MseI* endonuclease in plasmid pVR-25 was achieved.

Unexpectedly, while the ER2566 host carrying the optimized *MseI* methylase (#9 above) and the *MseI* endonuclease in plasmid pVR-25 expressed *MseI* endonuclease when first transformed and grown, the *MseI* was not stably maintained when this construct was stored in glycerol at -70°C.

The *MseI* methylase construct was further modified to achieve greater *MseI* modification of the host. As described above, the attempts to place the *MseI* methylase under the highest two levels of constitutive expression failed, presumably due to instability from high levels of methylation in the cells. To achieve a maximum tolerated level of methylation, a new M.*MseI* expression plasmid, pVR-26, was constructed. pVR-26 was constructed by inserting a second promoter, derived as described in (3) above (see Table 1).

This was done by cutting out a 1.244-kb DNA fragment containing the M. *MseI* coding region (*mseIM* gene) and upstream promoter from plasmid pNKR1707mseIM-9 (digested with *PmeI* and *MfeI*) and inserting it just downstream of the  $P_{lacUV5}$  promoter in vector pNEB193 (New England Biolabs, Inc., Beverly, MA) cut with *EcoRI* and *HincII*. Another *MseI* methylase construct, pVR-27, was made by deleting a 0.379-kb *PmeI-AfIII* fragment containing the  $P_{lacUV5}$  promoter and

TABLE 1. Summary of plasmids and *Escherichia coli* hosts used for optimizing recombinant *Msel* production



*lacI* operator from pVR-26. The pVR-26 *mseIM* methylase expressing vector allowed the stable expression of *MseI* endonuclease.

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## EXAMPLE V

### Optimization of the *MseI* restriction endonuclease expression

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#### 1) Expression vector construction

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As known very well in the art, restriction endonucleases are cytotoxic proteins. Attempting to clone a toxic gene into a plasmid designed to facilitate high expression is, in many cases, extremely difficult. One especially preferred plasmid for expressing cytotoxic genes is pLT7K (Kong, et al., *Nucl. Acids Res.* 28:3216-3222 (2000)). This plasmid contains the segment encoding replicative function (ori), a gene encoding β-lactamase, and a gene encoding kanamycin resistance which is flanked by restriction endonuclease sites suitable for cloning. The cI857 gene encodes a mutant form of the lambda bacteriophage repressor protein, which conditionally binds to DNA sequences (the CI operator) that overlap PL and PR (the lambda bacteriophage major leftward and rightward promoters, respectively). The lacI gene encodes a repressor protein, LacI, that conditionally binds a DNA sequence (the lac operator) which has been constructed to overlap PT7 (bacteriophage T7 RNA polymerase transcriptional promoter). Briefly, at high temperature (42°C) without IPTG, the

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antisense promoter is active, while  $P_{T7}$  is repressed by LacI. At 30°C and with IPTG, expression occurs from  $P_{T7}$ .

To adapt the pLT7K for overexpression of *MseI* restriction endonuclease gene, an NdeI restriction endonuclease site and ribosome binding site were introduced. Additionally, the coIE1 replicon was changed to the p15A replicon and copy number was decreased 3 times (from ~50 to ~15). To accomplish this, pLT7K was digested with *Ac*/I and *Bam*HI. The resulting 1.2-kb fragment containing cI857, the lambda PL, Kn resistance gene and the T7 promoter was isolated from an agarose gel using Qiagen QIAquick Gel Purification Kit (Cat. No. 28704) and ligated into pACYC184-T7ter $\Delta$ PshAI vector that was previously digested with *Cla*I and *Bam*HI. The pACYC184-T7ter $\Delta$ PshAI is a *Psh*AI deletion derivative of pACYC184-T7ter. This construct was designated pVR-24 (Fig. 11).

The open reading frame (ORF) for the *mseIR* gene was amplified by PCR with a set of forward (5' AGACTCCCCCATAT  
20 GACCCACGAACCGACGGATG 3' (SEQ ID NO:20) and reverse (5'  
GGGTGGTCCCGCTAGCTATTAGTAGGGACCGGGG 3' (SEQ ID  
NO:21) primers, where the underlined bases show the  
positions of the NdeI cleavage site for the forward primer.  
25 PCR was performed using Vent® DNA polymerase, 1X ThermoPol buffer, 500 ng of *Micrococcus* species (NEB#446) chromosomal DNA as the template in a 100  $\mu$ l PCR reaction, and primers. Twenty-five cycles were done consisting of 15

sec at 95°C, 60 sec at 68°C and 45 sec at 72°C. The resulting 700 bp PCR product was purified using a QiaQuick PCR purification protocol, treated with Klenow fragment, digested by *NdeI*, and purified once again using the QiaQuick PCR purification protocol.

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The resultant 700-bp *NdeI*-Blunt end fragment, containing *MseI* restriction endonuclease gene, was ligated into pVR-24 vector digested with *NdeI* and *StuI* and ligation mixture was transformed into *E. coli* ER2502 cells, previously modified with the *MseI* methylase gene construct pNKR1707*MseIm*-9. Out of 18 individual transformants analyzed, three contained *mseIR* gene. After sequencing the DNA insert containing *MseI* restriction endonuclease gene, one recombinant plasmid, pVR-25, was selected for producing the *MseI* restriction endonuclease.

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## 2) Strain Construction

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To increase LacI repressor copy number in the host, the strain ER2833 (T7lacIq strain) was constructed as described in U.S. Application Serial No. \_\_\_\_\_.

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3) Optimization of *MseI* restriction endonuclease overexpression in *E. coli* combining different hosts and plasmids expressing different levels of *MseI* methylase.

For optimization of *MseI* restriction endonuclease overexpression in *E. coli*, the pVR-25 plasmid was transferred

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into the expression strain ER2566/pCEF-8, which was pre-protected against *MseI* endonuclease auto-digestion by carrying one of these *MseI* methylase expressing plasmids (pNKR1707*MseIm*-9, pCR-26 and pVR-27). ER2566/pCEF-8 is a host strain containing a chromosomal copy of the gene for T7 RNA polymerase under control of the inducible lac promoter and a pSYX20 based plasmid, pCEF-8, which specifies low levels of T7 lysozyme, a natural inhibitor of T7 RNA polymerase. For additional information, see Moffatt, B. A., and Studier, F. W., "T7 Lysozyme inhibits transcription by T7 RNA polymerase," *Cell*, 49:221-227 (1987). In uninduced cells, lysozyme reduces the basal activity of the T7 RNA polymerase and increases the range of target genes that can be stably maintained in the expression host. In addition, another expression strain, ER2833/ pCEF-8 was used, which has an 10 copy of lacIq gene on the F' episome.

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Overall, six strains were used of *MseI* restriction endonuclease expression studies in *E. coli* (Table 1). All strains contain pVR-25 plasmid, expressing *MseI* restriction endonuclease, and pCEF-8 plasmid which encodes a T7 bacteriophage lysozyme gene. A variety of growth conditions were employed to grow transformed host cells to select for higher yields of *MseI* restriction endonuclease. The preferred medium in optimization experiments was Luria-Bertani (supplemented with 1 gram glucose and 1 gram MgCL2 per liter; subsequently referred to as supplemented LB) media.

The growth conditions were as follows:

MseRM1: cells from an individual colony were grown in 0.5 liter of LB medium at 42°C for 8h, after which IPTG was added to 0.2 mM final concentration to induce the T7 RNA polymerase and cells were grown overnight (15 h) at 30°C. Antibiotics were added as needed: 30 µg of kanamycin per ml, 100 µg of ampicillin per ml, and 30 µg of chloramphenicol per ml. Finally, cultures were harvested by centrifugation and frozen at -20°C.

MseRM3: for each experiment, cells from an individual colony were grown in 0.5 liter of LB medium at 30°C overnight (17 h), after which IPTG was added to 0.2 mM final concentration to induce T7 RNA polymerase and cells continued to grow for 4 h. Antibiotics were added as needed: 30 µg of kanamycin per ml, 100 µg of ampicillin per ml, and 30 µg of chloramphenicol per ml. Finally, cultures were harvested by centrifugation and frozen at -20°C.

MseRM4, MseRM5 and MseRM6: bacterial cultures were kept as frozen stock solutions at 70°C in 50% glycerol. Cultures used for seed inoculation were streaked onto LB medium plates containing the appropriate antibiotics to obtain single colonies. An individual colony was resuspended in 1 ml of LB medium and inoculated into a 1000-ml flasks containing 500 ml of LB medium supplemented with 30 µg of kanamycin/ml 100 µg of ampicillin/ml, and 30 µg of

chloramphenicol/ml. Cells were grown overnight (16 h) in a shaking incubator at 37°C and 250 rpm. Thereafter, IPTG was added to a final concentration of 0.2 mM. Cells were cultivated for another 4 h and then were harvested by centrifugation at 5 8,000 g for 5 min at 4°C and frozen at -20°C.

Two preferred restriction endonuclease assays for identifying high-level expression clones were used.

10            Sonication method: induced cultures (500 ml) were harvested and resuspended in 20 ml sonication buffer containing 10 mM Tris.HCl (pH 7.5) and 1 mM EDTA. Cells were sonicated on ice by four 30 second blasts with a macro-tip probe. A portion of the crude extract was added to lambda DNA (1µl) in NEBuffer 2 buffer (50 µl) and incubated for 1 hour at 37°C. DNA was fractionated by 0.8% gel electrophoresis and visualized by EtBr staining.

15            EXPRESS method: one ml of an overnight or induced culture (10 - 500 ml) was harvested and resuspended in 0.2 ml buffer containing 50 mM TRIS-HCl, pH 7.5 and 25% (vol/vol) sucrose and mixed until the solution was homogenous. 11 µl of 200 mM EDTA, pH 8.0 plus 200 µl of freshly-prepared 10 mg/ml lysozyme in 0.25M Tris-HCl (pH 8.0) were added and the solution was incubated on ice for 5 min. 11.5 µl of 1 M MgCl<sub>2</sub> and 24.2 µl of 5% (vol/vol) Brij-58 were then added. 20            The solution was gently mixed and incubated in room temperature for 15 min. After incubation the crude cell lysate

was centrifuged at maximum speed in a microcentrifuge for 15 min at 4°C. The supernatant was pipetted off into a new eppendorf tube and stored on ice until needed. Lambda DNA substrate (1.0 µg) was digested in *MseI* reaction buffer buffer (NEBuffer 2) with serial dilutions of cell extract for 1 hour at 37°C degree. DNA was fractionated by electrophoresis and visualized by EtdBr staining. Activity was determined by the presence of the appropriate size bands associated with a *MseI* digestion of lambda DNA.

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The results of optimization of *MseI* restriction endonuclease expression are summarized in Table 2.

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*MseRM1* strain gave a variable yield of *MseI* restriction endonuclease ( $0.08\text{-}0.5 \times 10^6$  U/g wet cells). Cells grew slowly and the lag time was exceptionally long.

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To enhance the stability and reproducibility of lac-based recombinant expression systems, the new host strain ER2833 (U.S. Application Serial No. \_\_\_\_\_) was constructed, which has an copy of *lacI<sup>q</sup>* gene on the F' episome. Indeed, the expression stability and plasmid maintenance in the *lacI<sup>q</sup>* host (*MseRM3*) was greatly enhanced: the yield of *MseI* restriction endonuclease was  $0.5\text{-}1.4 \times 10^6$  U/g wet cells. The *MseI* restriction endonuclease purified from this strain (see Example VI) was substantially free of non-specific endonuclease and exonuclease and the final yield was

Table 2. Summary of optimization of MseI restriction endonuclease expression in *E.coli*

Strain	Induction Conditions	Yield (U/g)	Comments
MseRM1	42°C 8 h (~40 Klett), shift to 30°C overnight	0.08-0.5x10 <sup>6</sup>	Difficult to repeat results. No activity from frozen culture
MseRM2			ER2566/pVR-26 grew very slowly, impossible to make competent cells
MseRM3	30°C 37°C overnight (~100 Klett), IPTG (0.2 mM) 4h	0.5-1.4x10 <sup>6</sup>	No activity from frozen culture. The enzyme prep gave ~150,000 U/g
MseRM4	37°C overnight (~100 Klett), shift to 30°C + IPTG (0.2 mM) 4h	3.3-8.6x10 <sup>6</sup>	This strain gave stable results from frozen culture, high MseI yield
MseRM5	37°C overnight (~100 Klett), shift to 30°C + IPTG (0.2 mM) 4h	1.5-3.4x10 <sup>6</sup>	This strain gave stable results from frozen culture, but has less MseI yield than MseRM4
MseRM6	37°C overnight (~100 Klett), shift to 30°C + IPTG (0.2 mM) 4h	3.3-3.8x10 <sup>6</sup>	This strain gave stable results from frozen culture, but has less MseI yield than MseRM4

<sup>a</sup>Resuspend an overnight colony (plated on 42°C) in 1ml LB, then add 0.1 ml of resuspended colony into the flask containing 500 ml of LB + antibiotic. Grow as described in EXAMPLE IV.

~150,000U/g. It is about 100 times greater yield than from native *Micrococcus* species (NEB#446).

Unfortunately, the MseRM3 strain showed no *MseI* restriction endonuclease activity after the strain was stored at -70 °C and revived. To solve this problem, the *MseI* methylase expression level was increased by constructing pVR-26 and pVR-27 plasmids (Example IV above). These strains (MseRM4, MseRM5 and MseRM6) gave high *MseI* restriction endonuclease yield from even after storing the strain at -70 °C and one strain, MseRM4 (NEB#1284; New England Biolabs, Inc., Beverly, MA) was used for scale-up in the 100 L production fermentor (see Example VI). The yield of *MseI* restriction endonuclease from this larger scale fermentation was  $0.5 \times 10^6$  U/g wet cells.

#### **EXAMPLE VI**

#### **Production of the recombinant *MseI* restriction endonuclease**

The *MseI* restriction enzyme was produced from recombinant *E. coli* strain NEB#1284 propagated to late-log phase in a 100-liter fermenter. A sample of these cells was deposited under the terms and conditions of the Budapest Treaty with the American Type Culture Collection on August 28, 2000 and received ATCC Accession No. PTA-2421.

## A) Cell Growth

The transformed *E. coli* host, NEB#1284 containing the recombinant *MseI* restriction endonuclease clone was stored as a frozen stock solution at -70°C in 50% glycerol. Cultures used for seed inoculation were streaked onto LB agar plates containing ampicillin (100µg/ml), chloramphenicol (30µg/ml) and kanamycin (50µg/ml) and incubated overnight at 37°C to obtain single colonies. Several colonies were used to inoculate 10 ml LB medium supplemented with 30 µg of kanamycin/ml 100 µg of ampicillin/ml, and 30 µg of chloramphenicol/ml. Cells were grown for 3 hrs in a shaking incubator at 37°C and 250 rpm and then at 30°C for an additional 3.5 hours (to avoid overgrowing the culture). The final corrected Klett of this culture was 122 or mid-log. This culture was used to inoculate 100-liter of LB supplemented with 30 µg of kanamycin/ml 100 µg of ampicillin/ml, and 30 µg of chloramphenicol/ml. The fermentation was run for 18 hours at 30°C with aeration of 2 SCFM (standard cubic feet per minute) and an agitation rate of 200 rpm. The final corrected Klett was 313. From this fermentation 331 grams of cells (wet weight) were harvested by continuous flow centrifugation and cells were stored at -70°C. A crude extract was made from 1 g of cells and the enzyme activity was estimated, using the method described above (see Example V). The yield of *MseI* restriction endonuclease in crude extract was 500,000# U/g, which is about 100 times more than in crude extract of *Micrococcus species* (NEB#446).

B) Purification of the *MseI* restriction endonuclease  
from NEB# 1284

5 All the following procedures were performed either on ice or at 4°C. 330 grams of cells were suspended in 990 ml Buffer A (0.15 M NaCl, 10mM Tris pH 7.5, 10 mM BME, 1 mM EDTA and 5% (v/v) glycerol and were broken by 4 passes at 12K through a Gaulin Press to an O.D. of 0.56. The 1150 ml supernatant was PEG precipitated by adding PEG 6000 to 7.5% and NaCl to 0.5 M and then incubated for 50 minutes at 4°C. The PEG slurry was centrifuged at 12K for 30 minutes at 4°C. The 580 ml of supernatant was diluted to 0.1M NaCl with Buffer A without NaCl and loaded onto a 430 ml Heparin

10 Hyper D column equilibrated with Buffer A. The column was washed with 1200 ml Buffer A and then a 4000 ml linear gradient from 0.1 M NaCl to 1.0 M NaCl was applied. The restriction enzyme activity eluted at 0.25-0.35M NaCl and was pooled. The Heparin Hyper D pool was diluted to 0.1M NaCl

15 with Buffer A without NaCl and loaded onto an 88 ml PEI column equilibrated with buffer A. The column was washed with 100 mls Buffer A and then a 1000 ml linear gradient from 0.1M to 1.7M NaCl was applied. The restriction enzyme activity eluted at 0.7 - 0.9M NaCl and was dialyzed against Buffer C

20 (50 mM NaCl, 15 mM Tris pH 7.5, 10 mM BME, 0.1 mM EDTA and 5% (v/v) glycerol) overnight and loaded onto a 20 ml

25 Source Q column equilibrated with Buffer C. The column was washed with 40 ml Buffer C and a 400 ml linear gradient from

0.05M NaCl to 1.0M NaCl was applied. The restriction enzyme activity eluted at 0.25M -0.35M NaCl and was pooled. The Source Q pool was dialyzed against Buffer D (10 mM KPO<sub>4</sub> pH7.0, 0.075M NaCl, 10 mM BME, 0.1mM EDTA, 5% (v/v) glycerol) and loaded onto a 20 ml Heparin TSK column equilibrated with Buffer D. The column was washed with 40 ml Buffer D and a 400 ml linear gradient from 0.075 M to 1 M NaCl in buffer D was applied. The restriction enzyme activity eluted at 0.3M -0.4M NaCl and was pooled. BSA was added to a final concentration of 100 µg/ml. The pool was dialyzed to Storage Buffer (20 mM Tris pH 7.5, 0.1M EDTA, 1mM DTT, 50 mM NaCl, 50% (v/v) glycerol, 200µg/ml BSA) overnight. This purification scheme yielded 26,000,000 units of *MseI* restriction endonuclease. The *MseI* restriction endonuclease obtained from this purification was substantially free of non-specific endonuclease and exonuclease.

The purity of the *MseI* restriction endonuclease preparation was checked by looking at the following criteria:

1. Ligation: After a 5-fold overdigestion of lambda DNA, greater than 95% of the DNA fragments produced were ligated with T4 DNA Ligase (at a 5' termini concentration of 1-2µM at 16°C. Of these ligated fragments, 95% were able to be recut.

2. Prolonged digestion: After incubating a 50µl reaction containing 1 µg of lambda and 100 units of enzyme

for 16 hours, the same banding pattern of DNA bands was produced as a reaction performed in one hour with one unit of enzyme.

5           3. Exonuclease Activity: After incubation of 100 units of enzyme for 4 hours at 37°C in a 50 µl reaction containing 1 µg sonicated  $^3\text{H}$  DNA ( $10^5$  cpm/µg) less than 0.4% radioactivity was released.

10           All tests were performed in the following reaction buffer: NEBuffer 2 (50 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, 1 mM DTT, (pH 7.9 at 25°C, supplemented with 100 µg/ml BSA. Unit determination: Lambda DNA substrate (1.0 µg) was digested in 50 µl 1X *Mse*I reaction buffer (NEBuffer 2) with serial dilutions of *Mse*I endonuclease for 1 hour at 37°C. DNA was fractionated by electrophoresis and visualized by EtdBr staining. Activity was determined by the presence of the appropriate size bands associated with a *Mse*I digestion of lambda DNA. One unit of restriction endonuclease activity is defined as the amount of enzyme required to completely digest 1 µg of substrate DNA in a total reaction volume of 50 µl in one hour using the NEBuffer specified.

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**WHAT IS CLAIMED IS:**

1. Isolated DNA coding for the *MseI* restriction endonuclease, wherein the isolated DNA is obtainable from *Micrococcus species.*

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2. A recombinant DNA vector comprising a vector into which a DNA segment coding for the *MseI* restriction endonuclease has been inserted.

15  
15  
3. Isolated DNA coding for the *MseI* restriction endonuclease and methylase, wherein the isolated DNA is obtainable from ATCC No. PTA-2421.

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20  
4. A cloning vector which comprises the isolated DNA of claim 3.

25  
25  
5. A host cell transformed by the cloning vector of claim 2 or 4.

6. A method of producing the *MseI* restriction endonuclease comprising culturing a host cell transformed with the vector of claim 2 or 4 under conditions suitable for expression of said endonuclease.

7. A method for producing a target restriction endonuclease, which method comprises:

- (a) isolating a gene coding for a protective modification methyltransferase;
- (b) obtaining an expression construct for the modification methyltransferase gene that allows complete protection of the host microorganism at substantially all growth phases, without leading to toxicity;

5 (c) isolating a gene for the cognate restriction endonuclease;

10 (d) placing the restriction endonuclease gene of step (c) into an expression vector; and

15 (e) combining the vector of step (d) with the vector of step (b) in a suitable host such that the combined vectors can be stably and reproducibly reisolated from storage in an expression-competent form.

20 8. The method of claim 7, wherein the growth phases examined of step (b) are selected from one or more of the group consisting of the logarithmic phase, stationary phase, a resting state achieved by starvation for carbon or nitrogen or other essential nutrient, a phase in which cells are in a special physiological state or in a phase in the presence of physiological insults.

25 9. The method of claim 7, in which step (b) comprises identifying regulatory elements capable of driving expression of the methyltransferase gene during said growth phases and placing these elements at an appropriate location in the expression construct.

10. The method of claim 9, in which selection is imposed by digesting pooled preparations of the vector with the target restriction endonuclease.

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11. The method of claim 7, in which the expression vector is selected from pUC19, pBluescript, pGEM, pRRS, pBR322 or from among moderate copy vectors selected from pACYC4, pSC101, or equivalents or from among unit copy vectors selected from F or pBELOBac11 or P1 or P1 or equivalents or from among vectors expressing target proteins from foreign promoters such as the T7 promoter selected from pET3a, pET21d or similar plasmids or vectors designed to provide very low basal expressions.

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15. The method of claim 11, in which the expression vector comprises a vector with low basal expression.

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20. The method of claim 12, in which the expression vector includes a T7 RNA polymerase promoter.

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25. The method of claim 13, in which the expression vector includes a lambda CI-regulates vector in the opposite sense to the expression promoter.

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15. The method of claim 14, in which the expression vector is pVR24.

## **ABSTRACT**

A method for cloning restriction-modification system is provided whereby the target modification methylase is produced and confers full protection during all growth phases in which the cognate restriction enzyme is present. The method is employed in the cloning of the *Mse*I restriction-modification system.

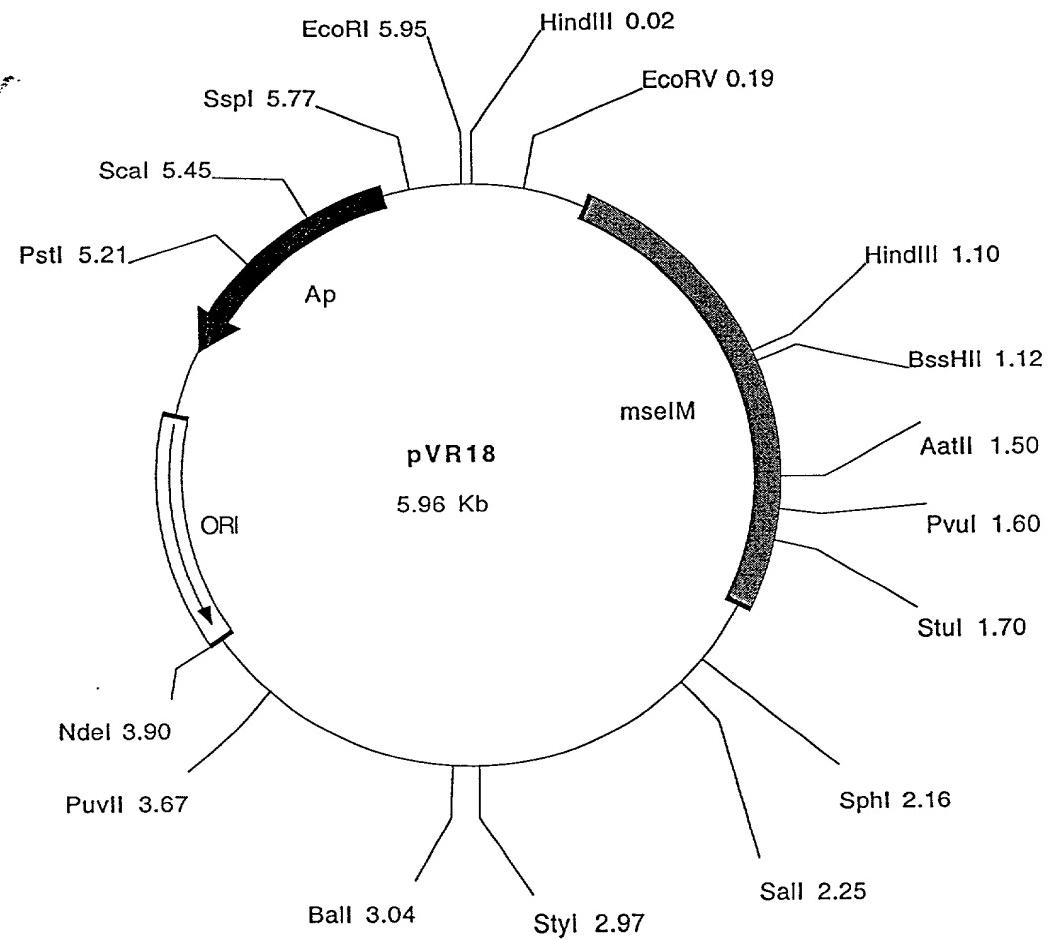
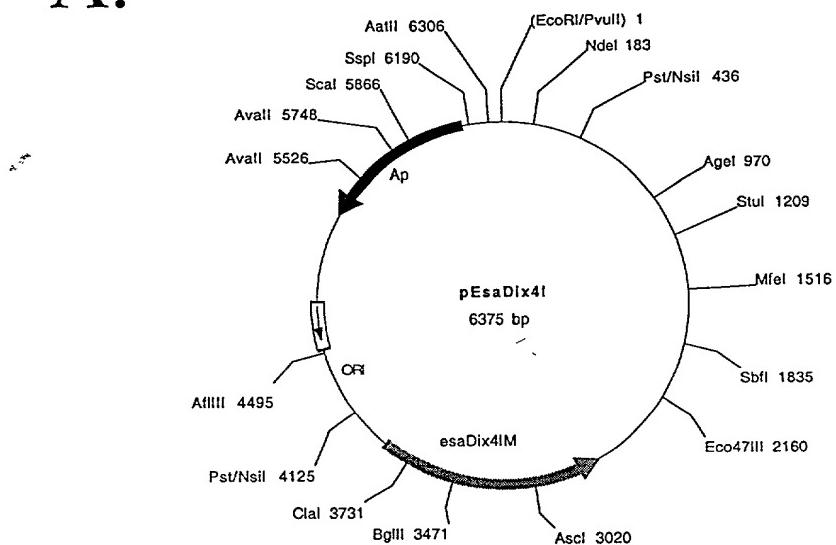


Fig. 1

A.



B.

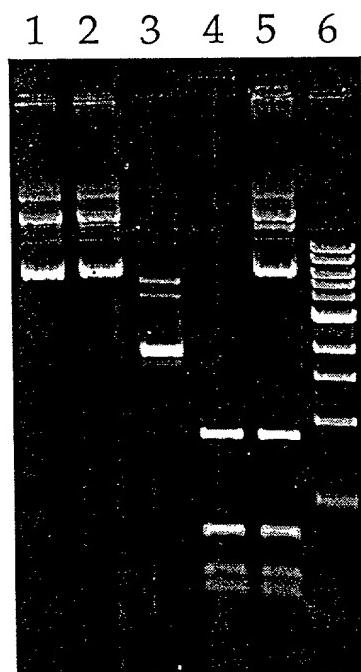
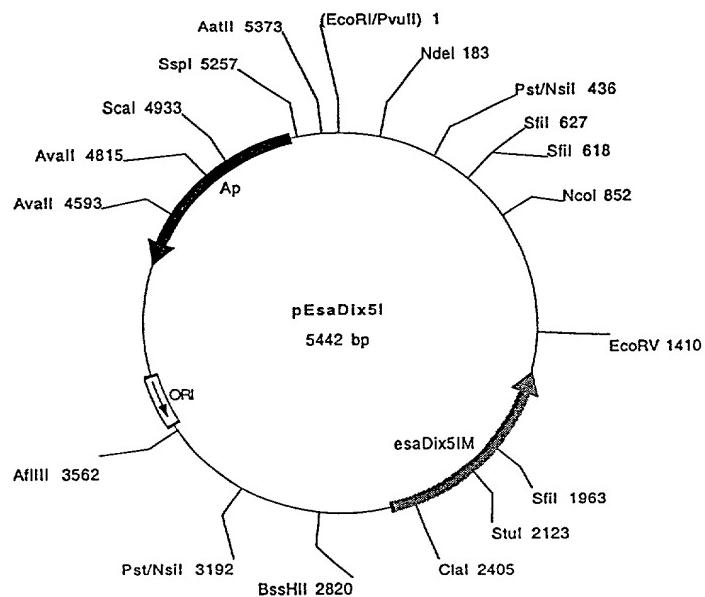


Fig 2

A.



B.

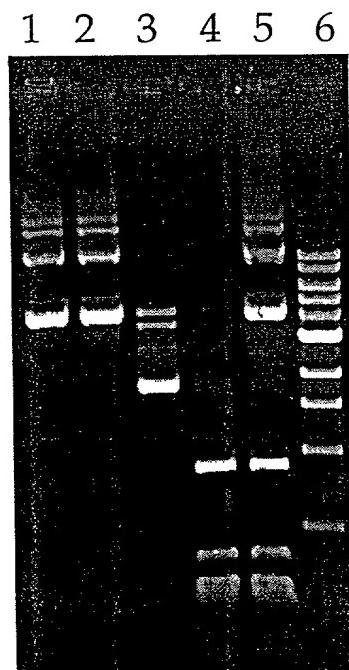


Fig. 3.

Muc 1 M

seq. ID NO: 1  
seq. ID NO: 2

1 ATGCCCTATCTGACCGTCTGGACGCCGGACGGAGACGACCTCATC  
M P I S T V W T P D G D D L I  
46 GTGGAGGCAGACAACCTCGATTTCAATTCAAACGCTCCCCGACCGCG  
V E A D N L D F I Q T L P D A  
91 AGCTTCCGAATGATCTACATCGATCCGCCGTTCAACACAGGGCGA  
S F R M I Y I D P P F N T G R  
136 ACGCAGCGGCTTCAGTCGCTCAAGACGACCCGCTCGGTACAGGG  
T Q R L Q S L K T T R S V T G  
181 TCGCGAGTCGGCTTCAAAGGCCAGACGTACGACACGGTCAAGAGC  
S R V G F K G Q T Y D T V K S  
226 ACTCTGCACTCGTATGACGACCGCTTCACCGACTATTGGTCGTT  
T L H S Y D D A F T D Y W S F  
271 CTCGAACCGCGTCTCCCTGGAGGCTTGGCGTTGCTCACCCCTGAC  
L E P R L L E A W R L L T P D  
316 GGCGCGCTCTATCTTCATCTGGATTACCGCGAGGTTCACTACGCC  
G A L Y L H L D Y R E V H Y A  
361 AAGGTCGTCCCTCGACGCGATGTTGGACCGAAAGCTTCCCTGAAC  
K V V L D A M F G R E S F L N  
406 GAGCTGATCTGGCGTACGACTACGGCGCGCCTCGAAGAGCAAG  
E L I W A Y D Y G A R S K S K  
451 TGGCCCACCAAGCACGACAACATCCTCGTGTATGTGAAGGACCCG  
W P T K H D N I L V Y V K D P  
496 AACAACTACGTCTGGAACGGTCAGGATGTAGATCGCGAGCCCTAC  
N N Y V W N G Q D V D R E P Y  
541 ATGGCGCCCGGGCTCGTTACACCCGAGAAGGTAGCGCTTGGCAAG  
M A P G L V T P E K V A L G K  
586 CTGCCACCACGCTGGTGACACAATCGTCCGCGTGCAGC  
L P T D V V W H T I V P P A S  
631 AAAGAGCGCACCGGGTACCGACACAGAACGCCGGTCGGCATCATC  
K E R T G Y A T Q K P V G I I  
676 CGTCGCATGATTCAAGCGAGCAGCAATGAAGGCGACTGGTTCTG  
R R M I Q A S S N E G D W V L  
721 GATTTCTCGCTGGTAGTGGACGCCGGCGCCGCCAG  
D F F A G S G T T G A A A R Q  
766 CTCGGACGCCGTTTGCTCGTAGACGTCAACCCAGAACGAAATC  
L G R R F V L V D V N P E A I  
811 GCGGTAATGGCAAAACGGTTGGATGACGGGGCATTGGACACCAGC  
A V M A K R L D D G A L D T S  
856 GTGACGATCGTCAGACTCCCCAGAGTGACCCACGAACCGACCGA  
V T I V Q T P Q S D P R T D G  
901 TGA 903

Fig. 4

esq Aix 4IM

SFQ 1D NO 3  
SFQ 1A NO 4

1 ATGCCCTACACTGGATTGGCCCGTAAACAGTTAACGCTTCCCACCA  
     M P T L D W P G K Q L S F P P  
 46 GCTACCTCCTTGATCTGGAGAGTGTGGTCACTGAGGGAGCGGAG  
     A T S L H L E S V V T E G A E  
 91 TCACCGCCTAATCGTCTGATTGGCGGACAACCTGCCGCTAATG  
     S P P N R L I W A D N L P L M  
 136 GTAGATTGTTGGCGAATATGAAGGAAAATCGATCTGATCTAC  
     V D L L A E Y E G K I D L I Y  
 181 GCCGATCCCCCTTTTTTACGGATCGTACCTATGCCGCCGAATT  
     A D P P F F T D R T Y A A R I  
 226 GGTCATGGGGAGGATTCGCGTCTGCACAAACCTGGCAGCTTGCA  
     G H G E D S R R P Q T W Q L A  
 271 GAAGGATATAACGGACGAGTGGAAAGGATTAGATGAATACTGGAC  
     E G Y T D E W K D L D E Y L D  
 316 TTCCCTTATCCACGCCCTGGTACTGATGTATCGACTGCTGGCACCA  
     F L Y P R L V L M Y R L L A P  
 361 CACGGAACGCTCTACTTGACCTGGACTGGCACGCCAATGCCCTAC  
     H G T L Y L H L D W H A N A Y  
 406 GTACGTGTACTGCTTGTGAGATCTCGGGCGACAGCGGTTCTC  
     V R V L L D E I F G R Q R F L  
 451 AACGAGATCGTCTGGATCTATCACGGCCCTCAGCCATCCGACGC  
     N E I V W I Y H G P S A I R R  
 496 GCCTTCAAGCGAAACATGATACCATCTGGTTATGTGAAAGGT  
     A F K R K H D T I L V Y V K G  
 541 GAAAACATACATTCAATGCGGATGCGGTTCTGCAACCTTACCAT  
     E N Y T F N A D A V R Q P Y H  
 586 CCGAGCACNCATAAGACCTTCGCTTCCTCCCCGAAGGCCGGCTTT  
     P S T H K T F A S S P K A G F  
 631 GGTAAGGTGCCGGATCTGCAGCGCGCAAAGTGGCCGAAGACTGG  
     G K V P D L Q R G K V P E D W  
 676 TGGTATTTCCGGCTGTGGCCCGTACACCGAGAACGGAGCGGC  
     W Y F P V V A R L H R E R S G  
 721 TATCCGACTCAAAGCCTCAAGCCTGCTGGAGCGGATCTGCTG  
     Y P T Q K P Q A L L E R I L L  
 766 GCCTCCTCGAACCGCAGCGATCTGGTGGCAGACTTCTCTGCC  
     A S S N A G D L V A D F F C G  
 811 TCAGGGACAACCGCTGTGGTGGCAGCCCGTCTGGACGGCGCTTC  
     S G T T A V V A A R L G R R F  
 856 CTGGTCAACGATGCAAGCTGGCGCGCCGTTCATGTGACACGCACA  
     L V N D A S W R A V H V T R T  
 901 CGCTTGCTACCGAGGGAGTAAGTTTCACTTTGAACGCCAGGAA  
     R L L R E G V S F T F E R Q E  
 946 ACTTTTACTCTACCTATCCAGCCACTTCCACCAAGATTGGTTGATC  
     T F T L P I Q P L P P D W L I  
 991 ATCGCCGAGGAGCAGATTGCCCTCCAAGCACCCCTTCGTTAGAT  
     I A E E Q I R L Q A P F L V D  
 1036 TTTGGGAAGTGGACGATCAATGGGATGGCAAAATCTCCGCAGC  
     F W E V D D Q W D G K I F R S  
 1081 CGTCATCAAGGCTTACGCTCCGCCCTCAGGAGCAGGCCGCTC  
     R H Q G L R S R L Q E Q A P L  
 1126 TCTCTACCATTGACCGGAAATGGACTGTTGTGTGACGGTAGTG  
     S L P L T G N G L L C V R V V  
 1171 AGCGTGAAGGGAATACTATGAGTTACAGGTGAGGCCGATAGC  
     S R E G E Y Y E F T G R A D S  
 1216 CCTCACCCGTATCGTTTG 1236  
     P H P V S F \*

Fig. 5.

esa DIX SIM

1 ATGATCACGAACCTGATGGAAAACGATGTCAATTGGCAAAATCTAC SFQ 1A : 5  
M I T N L M E N D V I G K I Y SFQ 1B : 6  
46 TTTGCCGACAACATGGAAGTCCTGCGAGGGCTTCCGGCGCGTCC  
F A D N M E V L R G L P A A S  
91 GTGGACCTGATCTACATCGATCCTCCGTTCAACACCGGAAAGGTT  
V D L I Y I D P P F N T G K V  
136 CAGGAGCGCACTCAGCTCAAAACGGTGCCTCCGAGTGGGGCGAT  
Q E R T Q L K T V R S E W G D  
181 CGCGTCGGATTCCAGGGCCGTCGCTACGAAAGCATCGTCGTGGGT  
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226 AAGAACGCTTACCGACTCTTCGACGACTATCTGGCTTTCTCG  
K K R F T D F F D D Y L A F L  
271 GAACCGCGCCTGGTCGAAGCCCATCGTGTCTGGCGCCGCACGGG  
E P R L V E A H R V L A P H G  
316 TGCCCTACTTTACGTCGACTACCGCGAGGTGCACTACTGTAAG  
C L Y F H V D Y R E V H Y C K  
361 GTCCTTCTGACGGCATCTCGGTGCGAGGCCCTTCTCAACGAG  
V L L D G I F G R E A F L N E  
406 ATCATCTGGGCCACGATTACGGCGGGCGTCCGAAGGACAGGTGG  
I I W A Y D Y G G R P K D R W  
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496 CGCCACGTGTTCAATGCGGACGAAATCGAGCGCATTCCCTACATG  
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901 TCCGTGCCGGGCCACCGGGTAA 924  
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Fig. 6 -

*nuc1 R*

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91 GACCCCTGGTCGGCAGCCCCCTCGAGTGGATCCTCGCGTTCCA  
D P W V G S P F E W I L R V P  
136 TCCAGAACGAAGGGCGCGGTGAGCTGCTCGTGAGCGAATGG  
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181 GCTAATGCCAAAGGCCTCCGTGTGAAGAGGTGGGTCCAGCGAT  
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A D R V I N G H R I E I K M S  
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T L W K S G G F K F Q Q I R D  
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Fig. 7.

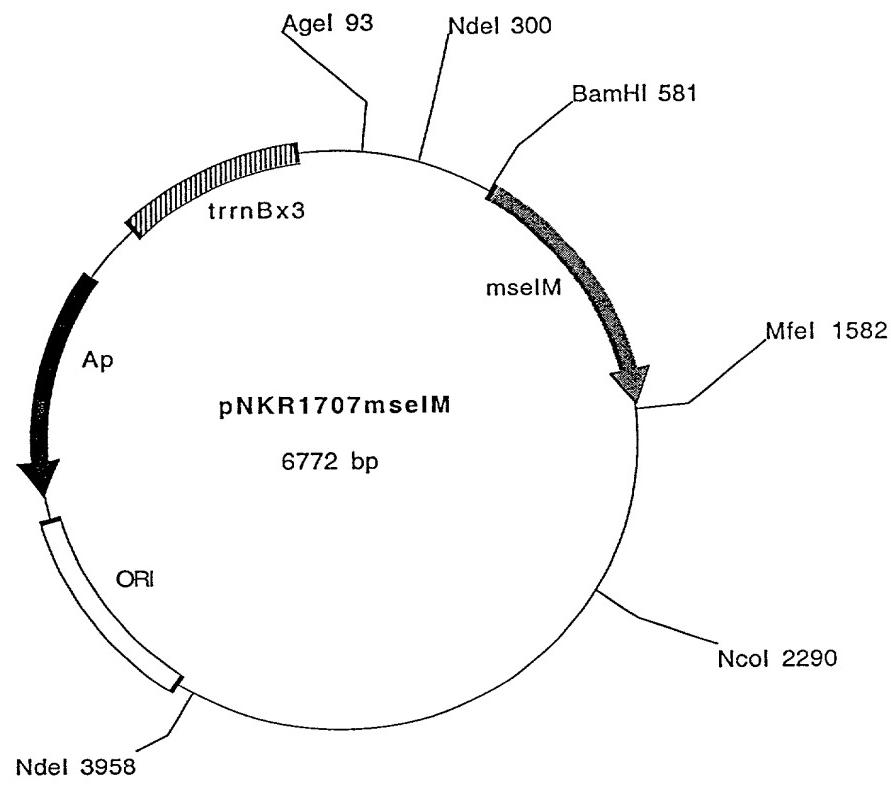
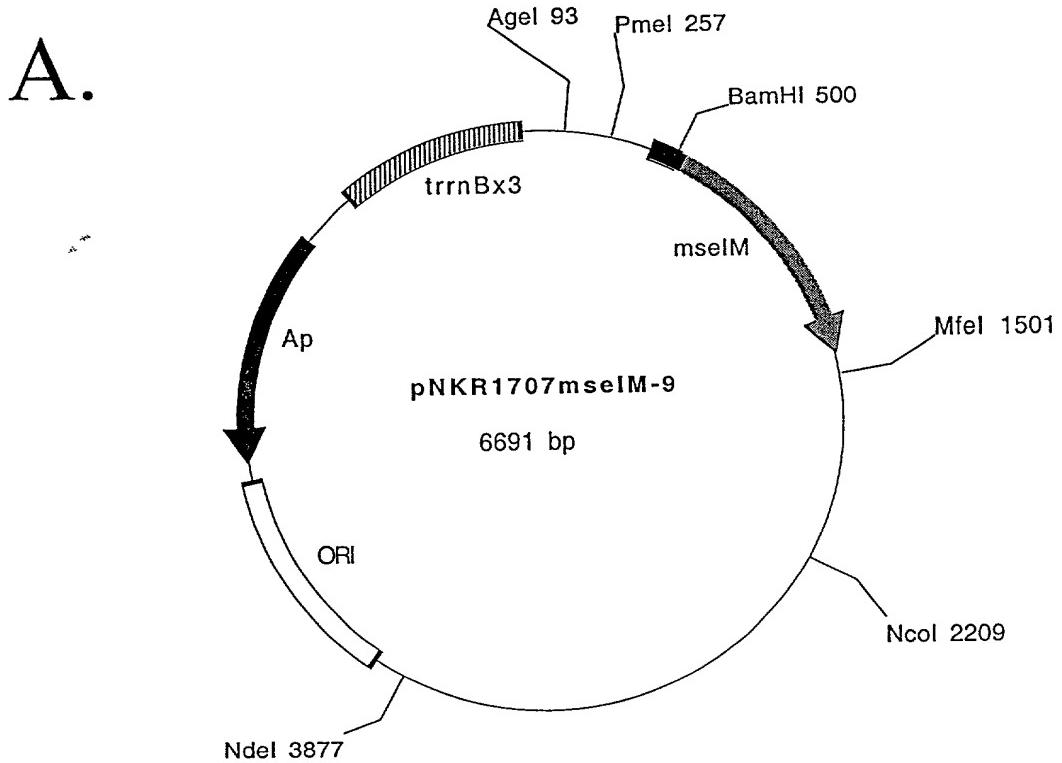


Fig. 8.



**B.**

*AgeI*

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*PmeI*

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-35 -10

- AGAATAAATTGTCGTGATT~~CAC~~TTAAATAAAAATTAAAGAGAAAAAAATTCTCT  
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*BamHI*

TGGATCC

Fig. 9.

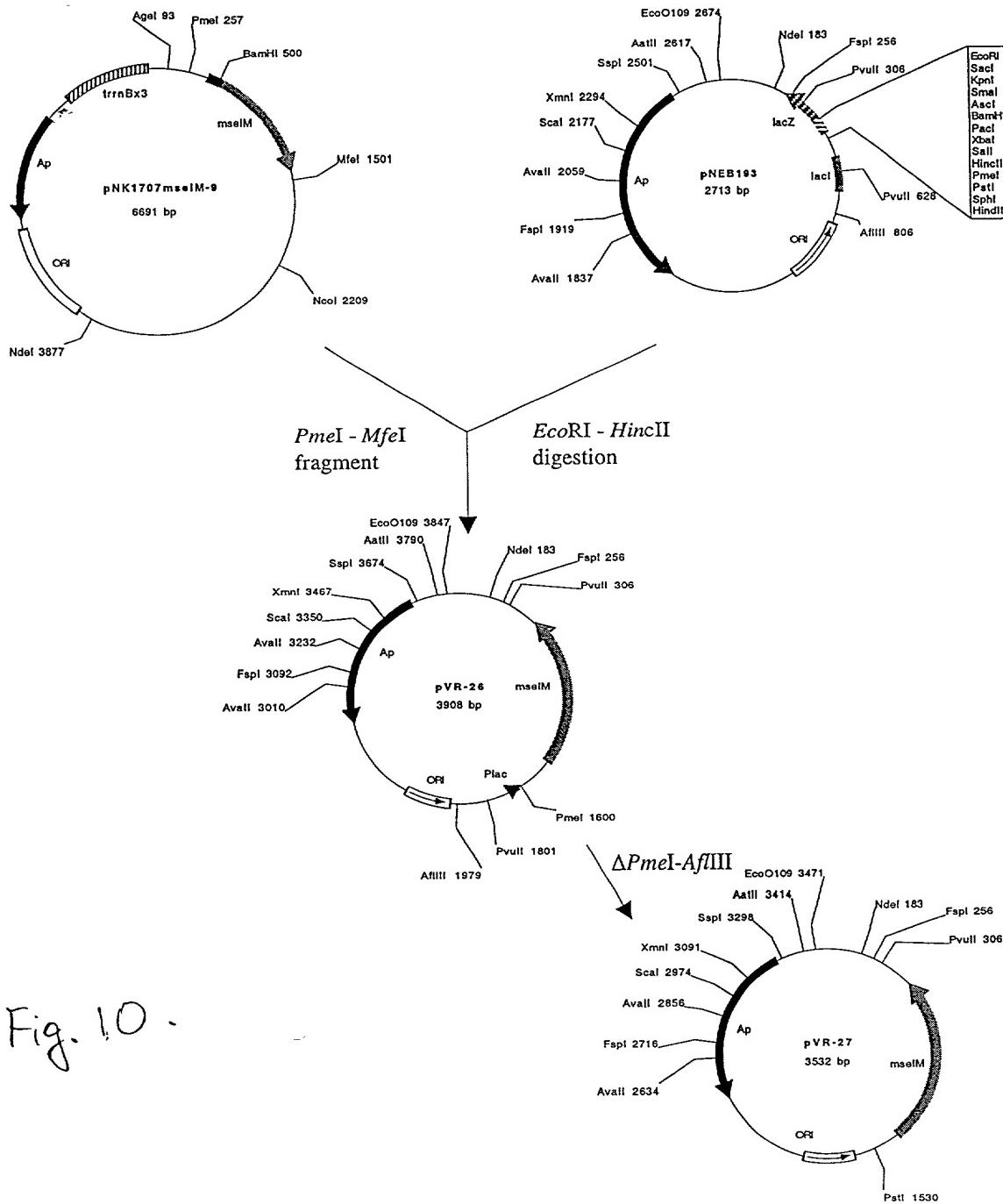


Fig. 10.

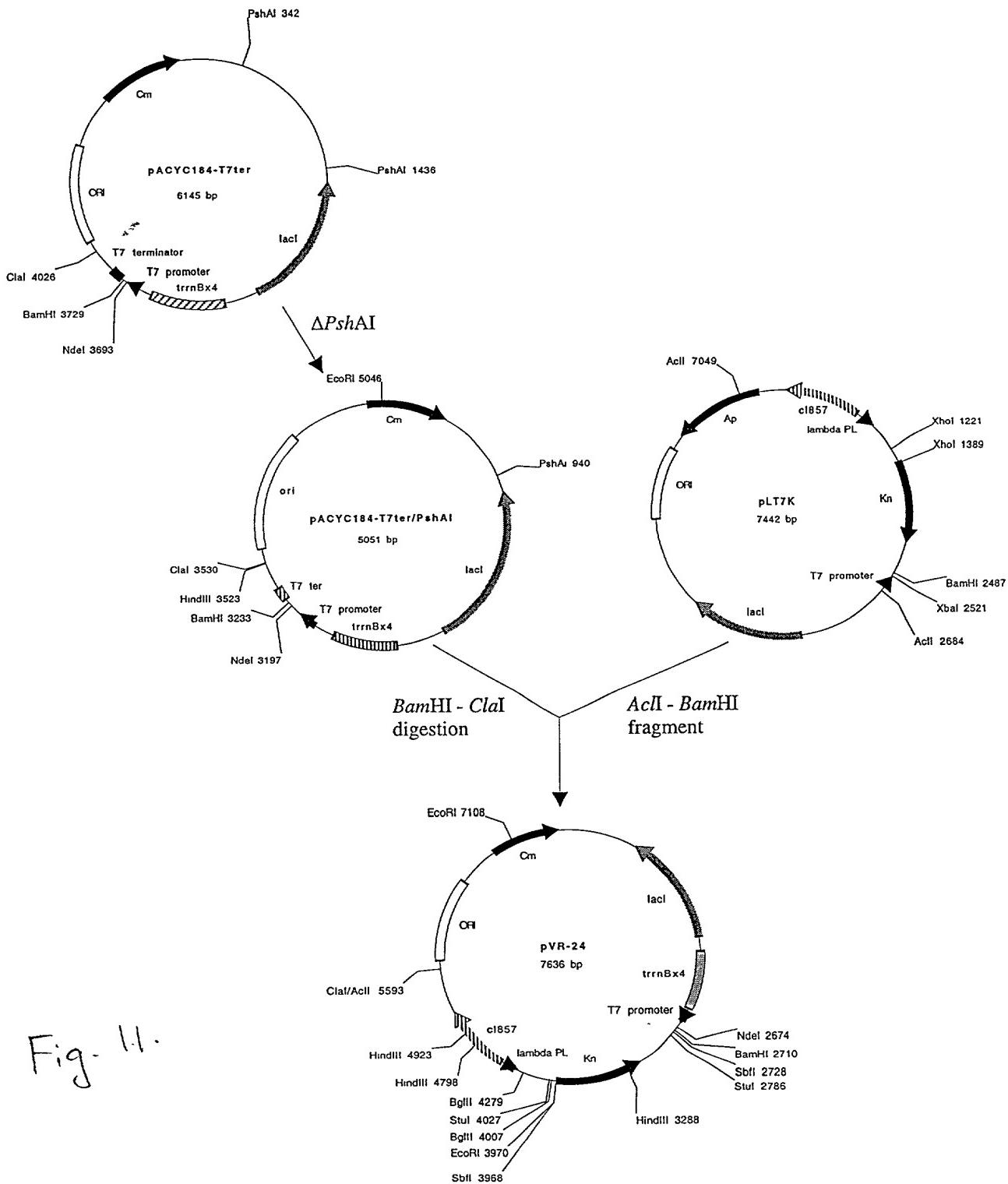
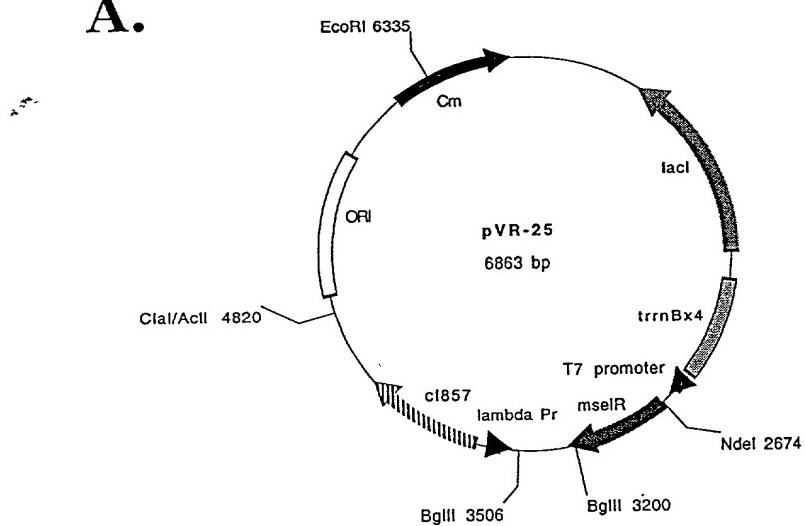
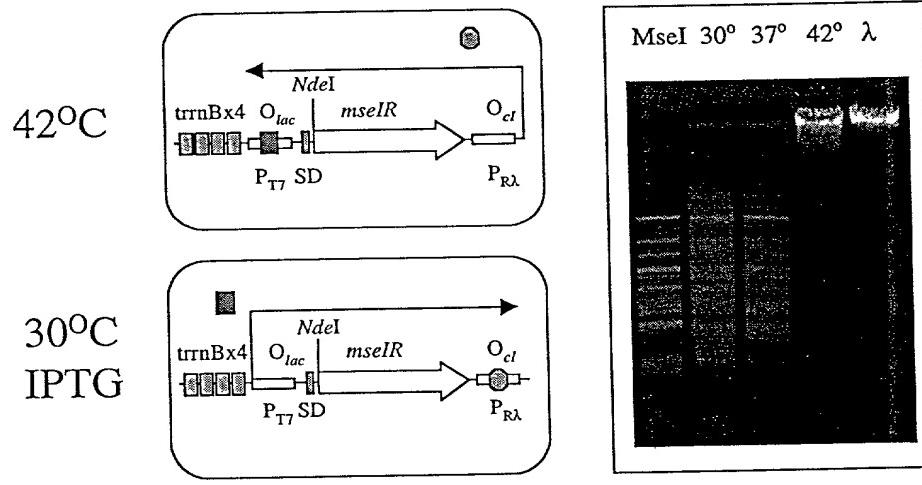


Fig. 11.

A.



B.



■ LacI  
○ Cl<sub>857</sub>

Fig. 12.

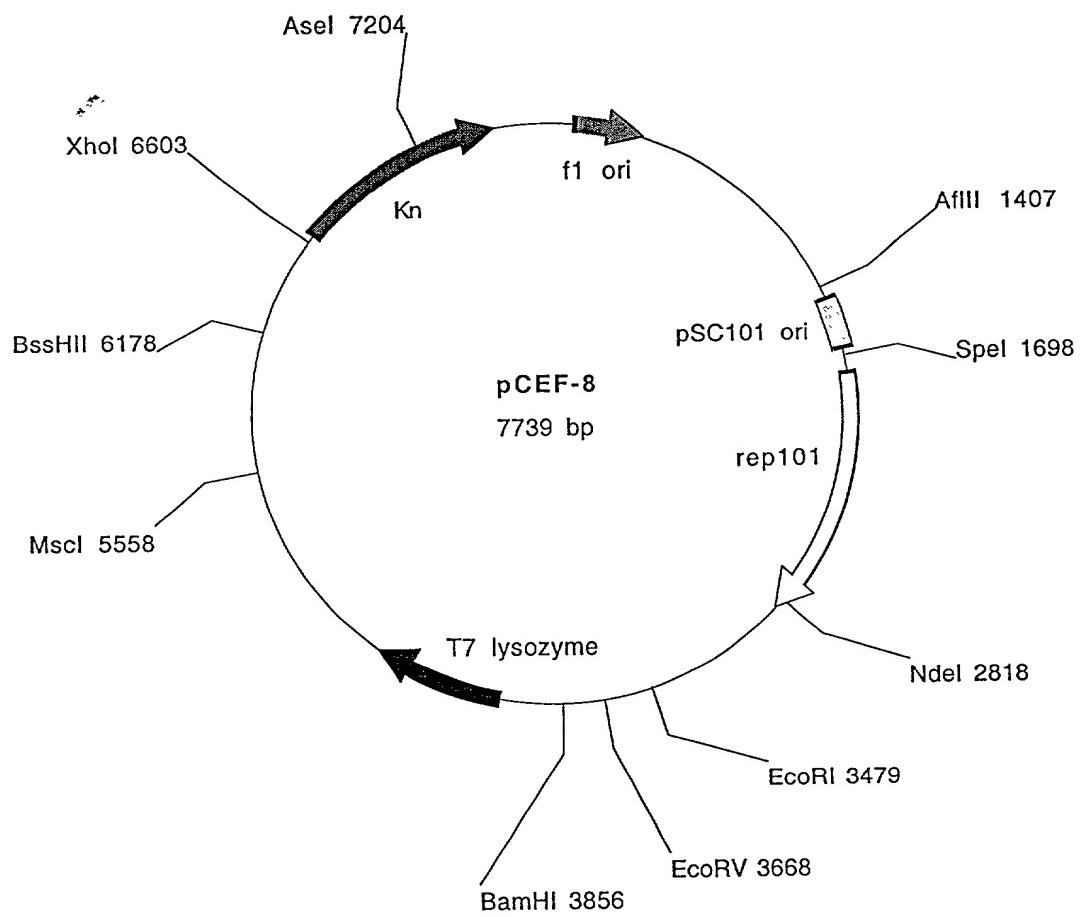
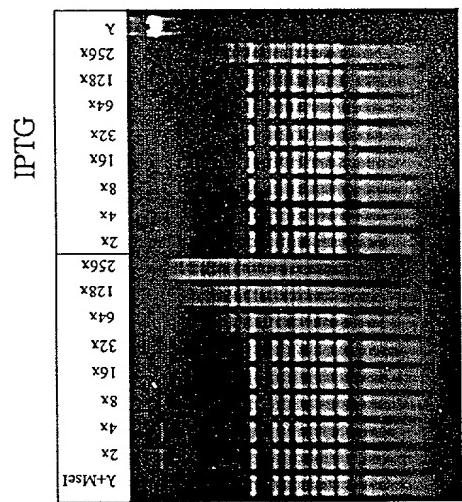
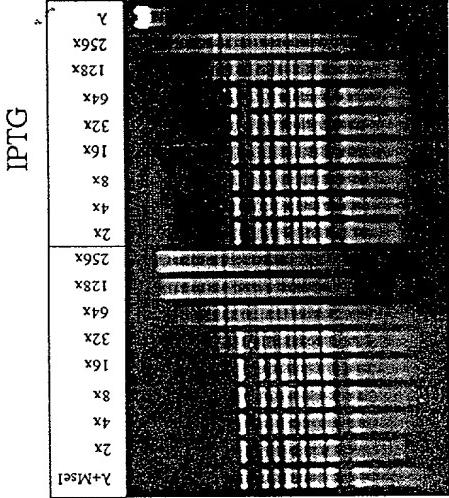


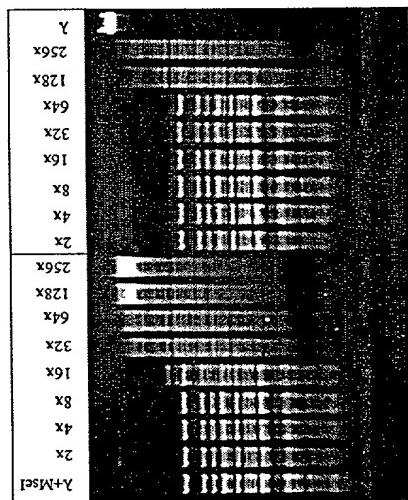
Fig. 13.



MseRM4



MseRM5



MseRM6

Tg. 14

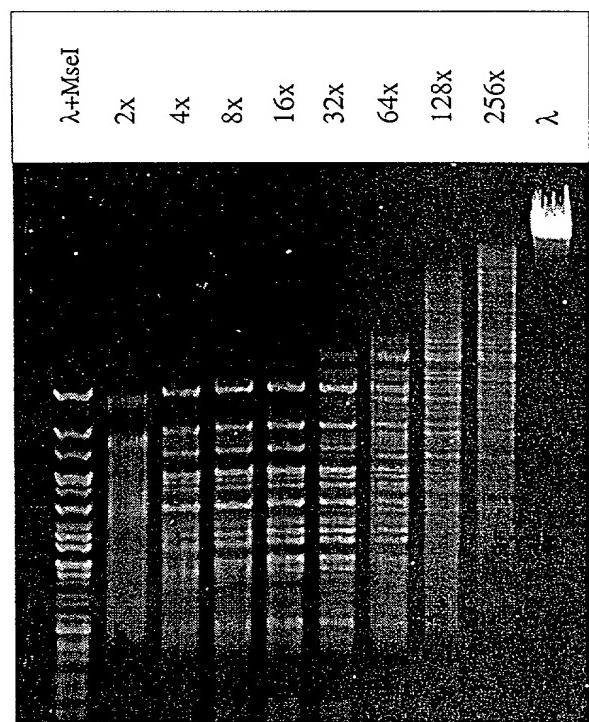


Fig. 15.

New England Biolabs, Inc.  
32 Tozer Road  
Beverly, MA 01915

DECLARATION  
AND POWER OF ATTORNEY  
Original Application

Attorney Docket No. NEB-181

As a below named inventor, I hereby declare that:

My residence, post address and citizenship are as stated below next to my name

I believe that I am the original, first and sole inventor (in only one name is listed at 201 below) or an original, first and joint inventor (if plural names are listed at 201-203 below) of the subject matter which is claimed and which a patent is sought on the invention entitled:

Method For Cloning And Producing The MseI Restriction Endonuclease

which is described and claimed in:

[X] the attached specification or [ ] the specification in Application Serial No. \_\_\_\_\_ filed \_\_\_\_\_  
(for declaration not accompanying application)  
And was amended on \_\_\_\_\_  
if applicable

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendments referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

FOREIGN APPLICATION(S) IF ANY, FILED WITHIN 12 MONTHS PRIOR TO THE FILING DATE OF THIS APPLICATION

COUNTRY	APPLICATION	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. 119
			YES NO
			YES NO

ALL FOREIGN APPLICATION(S) IF ANY, FILED MORE THAN 12 MONTHS PRIOR TO THE FILING DATE OF THIS APPLICATION

COUNTRY	APPLICATION	(day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. 119
π			

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (Patented, Pending, Abandoned)

DECLARATION  
AND POWER OF ATTORNEY  
PAGE 2 OF 3

**POWER OF ATTORNEY:**

As a named inventor, I hereby appoint the following attorney with full powers of association, substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

Gregory D. Williams  
(Registration No. 30901)

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Beverly, MA 01915

**DIRECT TELEPHONE CALLS TO:**

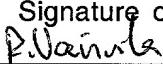
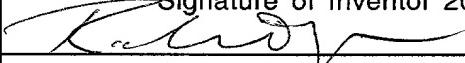
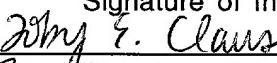
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General Counsel  
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Fax: (978) 927-1705

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4	Post Office Address	Post Office Address 29 Arlington Avenue	City/State/Country Beverly, MA	Zip Code 01915
2	Full Name of Inventor	Last Name Raleigh	First Name Elisabeth	Middle Name A.
0	Residence & Citizenship	City Somerville	State/Foreign Country Massachusetts	Citizenship US
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DECLARATION  
AND POWER OF ATTORNEY  
PAGE 3 OF 3

2	Full Name of Inventor	Last Name	First Name	Middle Name
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6	Post Office Address	Post Office Address	City/State/Country	Zip Code
2	Full Name of Inventor	Last Name	First Name	Middle Name
0	Residence & Citizenship	City	State/Foreign Country	Citizenship
7	Post Office Address	Post Office Address	City/State/Country	Zip Code
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2	Full Name of Inventor	Last Name	First Name	Middle Name
0	Residence & Citizenship	City	State/Foreign Country	Citizenship
9	Post Office Address	Post Office Address	City/State/Country	Zip Code

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature of Inventor 201 	Date 11 Oct 00
Signature of Inventor 202 	Date 3 Oct 00
Signature of Inventor 203 	Date 12 Oct 00
Signature of Inventor 204 	Date 11 Oct 00
Signature of Inventor 205 	Date 11 Oct 00
Signature of Inventor 206	Date
Signature of Inventor 207	Date
Signature of Inventor 208	Date
Signature of Inventor 209	Date

Docket No.: NEB-181

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: R. Vaisvila, et al. EXAMINER:

SERIAL NO.: GROUP:

FILED:

FOR: Method For Cloning And Producing The *MseI*  
Restriction Endonuclease

Hon. Commissioner of Patents  
and Trademarks

Sir:

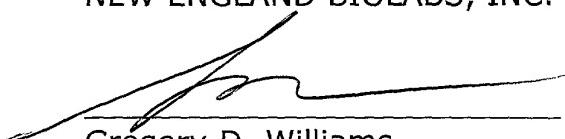
**STATEMENTS IN SUPPORT OF FILING  
AND SUBMISSIONS IN ACCORDANCE WITH 37 C.F.R.  
§§1.821.1-1.825**

In accordance with 37 C.F.R. §§1.821-1.825, I hereby state that the contents of the paper and computer-readable copies of the sequence listing submitted in accordance with 37 C.F.R. §1.821(c) and (e), respectively, are the same. I hereby state that the submission, filed in accordance with 37 C.F.R. §1.821(g) does not introduce new matter.

Respectfully submitted,

NEW ENGLAND BIOLABS, INC.

Date: 10/12/01



Gregory D. Williams  
(Reg. No.: 30901)  
Attorney for Applicant  
32 Tozer Road  
Beverly, MA 01915

SEQUENCE LISTING

<110> VAISVILA, ROMUALDAS  
MORGAN, RICHARD D.  
KUCERA, REBECCA B.  
CLAUS, TOBY E.  
RALEIGH, ELISABETH A.

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ENDONUCLEASE

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Thr Ser Leu His Leu Glu Ser Val Val Thr Glu Gly Ala Glu Ser Pro  
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Ser Arg Arg Pro Gln Thr Trp Gln Leu Ala Glu Gly Tyr Thr Asp Glu			
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tgg aag gat tta gat gaa tac ctg gac ttc ctt tat cca cgc ctg gta			336
Trp Lys Asp Leu Asp Glu Tyr Leu Asp Phe Leu Tyr Pro Arg Leu Val			
100	105	110	
ctg atg tat cga ctg ctg gca cca cac gga acg ctc tac ttg cac ctg			384
Leu Met Tyr Arg Leu Leu Ala Pro His Gly Thr Leu Tyr Leu His Leu			
115	120	125	
gac tgg cac gcc aat gcc tac gta cgt gta ctg ctt gat gag atc ttc			432
Asp Trp His Ala Asn Ala Tyr Val Arg Val Leu Leu Asp Glu Ile Phe			
130	135	140	
ggg cga cag cgg ttt ctc aac gag atc gtc tgg atc tat cac ggc ccc			480
Gly Arg Gln Arg Phe Leu Asn Glu Ile Val Trp Ile Tyr His Gly Pro			
145	150	155	160
tca gcc atc cga cgc gcc ttc aag cgc aaa cat gat acc atc ttg gtt			528
Ser Ala Ile Arg Arg Ala Phe Lys Arg Lys His Asp Thr Ile Leu Val			
165	170	175	
tat gtg aaa ggt gaa aac tat aca ttc aat gcg gat gcg gtt cgt caa			576
Tyr Val Lys Gly Glu Asn Tyr Thr Phe Asn Ala Asp Ala Val Arg Gln			
180	185	190	
cct tac cat ccg agc acn cat aag acc ttc gct tcc tcc ccg aag gcc			624
Pro Tyr His Pro Ser Xaa His Lys Thr Phe Ala Ser Ser Pro Lys Ala			
195	200	205	
ggc ttt ggt aag gtg ccg gat ctg cag cgc ggc aaa gtg ccc gaa gac			672
Gly Phe Gly Lys Val Pro Asp Leu Gln Arg Gly Lys Val Pro Glu Asp			
210	215	220	

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Trp Trp Tyr Phe Pro Val Val Ala Arg Leu His Arg Glu Arg Ser Gly	
225 230 235 240	
tat ccg actcaa aag cct caa gcc ttg ctg gag cgg atc ctg ctg gcc	768
Tyr Pro Thr Gln Lys Pro Gln Ala Leu Leu Glu Arg Ile Leu Leu Ala	
245 250 255	
tcc tcg aac gca ggc gat ctg gtg gca gac ttc ttc tgc ggc tca ggg	816
Ser Ser Asn Ala Gly Asp Leu Val Ala Asp Phe Phe Cys Gly Ser Gly	
260 265 270	
aca acc gct gtg gtg gca gcc cgt ctg gga cgg cgc ttc ctg gtc aac	864
Thr Thr Ala Val Val Ala Ala Arg Leu Gly Arg Arg Phe Leu Val Asn	
275 280 285	
gat gca agc tgg cgc gcc gtt cat gtg aca cgc aca cgc ttg cta cgc	912
Asp Ala Ser Trp Arg Ala Val His Val Thr Arg Thr Arg Leu Leu Arg	
290 295 300	
gag gga gta agt ttc act ttt gaa cgc cag gaa act ttt act cta cct	960
Glu Gly Val Ser Phe Thr Phe Glu Arg Gln Glu Thr Phe Thr Leu Pro	
305 310 315 320	
atc cag cca ctt cca cca gat tgg ttg atc atc gcc gag gag cag att	1008
Ile Gln Pro Leu Pro Pro Asp Trp Leu Ile Ile Ala Glu Glu Gln Ile	
325 330 335	
cgc ctc caa gca ccc ttt ctc gta gat ttt tgg gaa gtg gac gat caa	1056
Arg Leu Gln Ala Pro Phe Leu Val Asp Phe Trp Glu Val Asp Asp Gln	
340 345 350	
tgg gat ggc aaa atc ttc cgc agc cgt cat caa ggc tta cgc tcc cgc	1104
Trp Asp Gly Lys Ile Phe Arg Ser Arg His Gln Gly Leu Arg Ser Arg	
355 360 365	
ctt cag gag cag gcg ccg ctc tct cta cca ttg acc ggg aat gga ctg	1152
Leu Gln Glu Gln Ala Pro Leu Ser Leu Pro Leu Thr Gly Asn Gly Leu	
370 375 380	
ttg tgt gta cgg gta gtg agc cgt gaa ggg gaa tac tat gag ttc aca	1200
Leu Cys Val Arg Val Val Ser Arg Glu Gly Glu Tyr Tyr Glu Phe Thr	
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Pro Asn Arg Leu Ile Trp Ala Asp Asn Leu Pro Leu Met Val Asp Leu  
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Leu Ala Glu Tyr Glu Gly Lys Ile Asp Leu Ile Tyr Ala Asp Pro Pro  
50 55 60  
  
Phe Phe Thr Asp Arg Thr Tyr Ala Ala Arg Ile Gly His Gly Glu Asp  
65 70 75 80  
  
Ser Arg Arg Pro Gln Thr Trp Gln Leu Ala Glu Gly Tyr Thr Asp Glu  
85 90 95  
  
Trp Lys Asp Leu Asp Glu Tyr Leu Asp Phe Leu Tyr Pro Arg Leu Val  
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Leu Met Tyr Arg Leu Leu Ala Pro His Gly Thr Leu Tyr Leu His Leu  
115 120 125  
  
Asp Trp His Ala Asn Ala Tyr Val Arg Val Leu Leu Asp Glu Ile Phe  
130 135 140  
  
Gly Arg Gln Arg Phe Leu Asn Glu Ile Val Trp Ile Tyr His Gly Pro  
145 150 155 160  
  
Ser Ala Ile Arg Arg Ala Phe Lys Arg Lys His Asp Thr Ile Leu Val  
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Tyr Val Lys Gly Glu Asn Tyr Thr Phe Asn Ala Asp Ala Val Arg Gln  
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Pro Tyr His Pro Ser Xaa His Lys Thr Phe Ala Ser Ser Pro Lys Ala  
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Gly Phe Gly Lys Val Pro Asp Leu Gln Arg Gly Lys Val Pro Glu Asp  
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Trp Trp Tyr Phe Pro Val Val Ala Arg Leu His Arg Glu Arg Ser Gly  
225                    230                    235                    240

Tyr Pro Thr Gln Lys Pro Gln Ala Leu Leu Glu Arg Ile Leu Leu Ala  
245                    250                    255

Ser Ser Asn Ala Gly Asp Leu Val Ala Asp Phe Phe Cys Gly Ser Gly  
260                    265                    270

Thr Thr Ala Val Val Ala Ala Arg Leu Gly Arg Arg Phe Leu Val Asn  
275                    280                    285

Asp Ala Ser Trp Arg Ala Val His Val Thr Arg Thr Arg Leu Leu Arg  
290                    295                    300

Glu Gly Val Ser Phe Thr Phe Glu Arg Gln Glu Thr Phe Thr Leu Pro  
305                    310                    315                    320

Ile Gln Pro Leu Pro Pro Asp Trp Leu Ile Ile Ala Glu Glu Gln Ile  
325                    330                    335

Arg Leu Gln Ala Pro Phe Leu Val Asp Phe Trp Glu Val Asp Asp Gln  
340                    345                    350

Trp Asp Gly Lys Ile Phe Arg Ser Arg His Gln Gly Leu Arg Ser Arg  
355                    360                    365

Leu Gln Glu Gln Ala Pro Leu Ser Leu Pro Leu Thr Gly Asn Gly Leu  
370                    375                    380

Leu Cys Val Arg Val Val Ser Arg Glu Gly Glu Tyr Tyr Glu Phe Thr  
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gcc gac aac atg gaa gtc ctg cga ggg ctt ccg gcg gcg tcc gtg gac 96  
Ala Asp Asn Met Glu Val Leu Arg Gly Leu Pro Ala Ala Ser Val Asp  
20 25 30

ctg atc tac atc gat cct ccg ttc aac acc gga aag gtt cag gag cgc 144  
Leu Ile Tyr Ile Asp Pro Pro Phe Asn Thr Gly Lys Val Gln Glu Arg  
35 40 45

act cag ctc aaa acg gtg cgc tcc gag tgg ggc gat cgc gtc gga ttc 192  
Thr Gln Leu Lys Thr Val Arg Ser Glu Trp Gly Asp Arg Val Gly Phe  
50 55 60

cag ggc cgt cgc tac gaa agc atc gtc gtg ggt aag aag cgc ttt acc 240  
Gln Gly Arg Arg Tyr Glu Ser Ile Val Val Gly Lys Lys Arg Phe Thr  
65 70 75 80

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Asp Phe Phe Asp Asp Tyr Leu Ala Phe Leu Glu Pro Arg Leu Val Glu  
85 90 95

gcc cat cgt gtt ctg gcg ccg cac ggg tgc ctc tac ttt cac gtc gac 336  
Ala His Arg Val Leu Ala Pro His Gly Cys Leu Tyr Phe His Val Asp  
100 105 110

tac cgc gag gtg cac tac tgt aag gtc ctt ctt gac ggc atc ttc ggt 384  
Tyr Arg Glu Val His Tyr Cys Lys Val Leu Leu Asp Gly Ile Phe Gly  
115 120 125

cgc gag gcc ttt ctc aac gag atc atc tgg gcc tac gat tac ggc ggg 432  
Arg Glu Ala Phe Leu Asn Glu Ile Ile Trp Ala Tyr Asp Tyr Gly Gly  
130 135 140

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Arg Pro Lys Asp Arg Trp Pro Pro Lys His Asp Asn Ile Leu Leu Tyr  
145 150 155 160

gcc aag act ccc ggt cgc cac gtg ttc aat gcg gac gaa atc gag cgc 528  
Ala Lys Thr Pro Gly Arg His Val Phe Asn Ala Asp Glu Ile Glu Arg  
165 170 175

att ccc tac atg gct ccg ggc ctg gtt ggc ccc gaa aag gca gcc cgt 576

Ile Pro Tyr Met Ala Pro Gly Leu Val Gly Pro Glu Lys Ala Ala Arg			
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gga aaa ctg cca acc gac acg tgg tgg cat acg atc gtt ccg acc agc	624		
Gly Lys Leu Pro Thr Asp Thr Trp Trp His Thr Ile Val Pro Thr Ser			
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ggc tcc gag aag acc ggg tat cca acc cag aaa cct tta ggg att ctc	672		
Gly Ser Glu Lys Thr Gly Tyr Pro Thr Gln Lys Pro Leu Gly Ile Leu			
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cgc cgt att gtg cag gca tcg tct cat ccg ggg gca gtc gtg ctc gac	720		
Arg Arg Ile Val Gln Ala Ser Ser His Pro Gly Ala Val Val Leu Asp			
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ttc ttc gcc ggc agt ggg aca aca ggg gta gcg gct ttt gag ttg ggc	768		
Phe Phe Ala Gly Ser Gly Thr Thr Gly Val Ala Ala Phe Glu Leu Gly			
245	250	255	
cgg cgt ttc att ctg gtc gat aac cat ccg gag gcc ctc cag gtg atg	816		
Arg Arg Phe Ile Leu Val Asp Asn His Pro Glu Ala Leu Gln Val Met			
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gcc agg cgc ttc gac ggc atc gag ggg atc gaa tgg gtg ggc ttc gat	864		
Ala Arg Arg Phe Asp Gly Ile Glu Gly Ile Glu Trp Val Gly Phe Asp			
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ccg aca ccg tac cag aag ggc gca aag cag cgc cgc tcc tgc ccg gcg	912		
Pro Thr Pro Tyr Gln Lys Gly Ala Lys Gln Arg Arg Ser Cys Pro Ala			
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Pro Thr Gly			
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Asp	Phe	Phe	Asp	Asp	Tyr	Leu	Ala	Phe	Leu	Glu	Pro	Arg	Leu	Val	Glu
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145														155	
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Ala	Lys	Thr	Pro	Gly	Arg	His	Val	Phe	Asn	Ala	Asp	Glu	Ile	Glu	Arg
165														175	
Ile	Pro	Tyr	Met	Ala	Pro	Gly	Leu	Val	Gly	Pro	Glu	Lys	Ala	Ala	Arg
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Arg	Arg	Ile	Val	Gln	Ala	Ser	Ser	His	Pro	Gly	Ala	Val	Val	Leu	Asp
225														240	
Phe	Phe	Ala	Gly	Ser	Gly	Thr	Thr	Gly	Val	Ala	Ala	Phe	Glu	Leu	Gly
245														255	
Arg	Arg	Phe	Ile	Leu	Val	Asp	Asn	His	Pro	Glu	Ala	Leu	Gln	Val	Met
260														270	
Ala	Arg	Arg	Phe	Asp	Gly	Ile	Glu	Gly	Ile	Glu	Trp	Val	Gly	Phe	Asp
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Pro Thr Gly  
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Ser Ala Ala Asn Leu Ala Asp Arg Tyr Val Ala Ser Glu Asp Asp Pro  
20 25 30

tgg gtc ggc agc ccg ttc gag tgg atc ctt cgc gtt cca tcc aga acg 144  
Trp Val Gly Ser Pro Phe Glu Trp Ile Leu Arg Val Pro Ser Arg Thr  
35 40 45

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Lys Gly Ala Val Gly Glu Leu Leu Val Ser Glu Trp Ala Asn Ala Lys  
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ggc ctc cgt gtg aag agg tcg ggg tcc agc gat gcg gac cgc gtg atc 240  
Gly Leu Arg Val Lys Arg Ser Gly Ser Ser Asp Ala Asp Arg Val Ile  
65 70 75 80

aac ggg cat cgc atc gag atc aag atg tcg act ttg tgg aag tcc ggc 288  
Asn Gly His Arg Ile Glu Ile Lys Met Ser Thr Leu Trp Lys Ser Gly  
85 90 95

ggc ttc aag ttt cag cag atc cgg gat cag gag tac gac ttt tgc ctc 336  
Gly Phe Lys Phe Gln Gln Ile Arg Asp Gln Glu Tyr Asp Phe Cys Leu  
100 105 110

tgc ctt ggg atc agc ccg ttc gaa gtg cac gcg tgg ctg ctg ccc aaa 384  
Cys Leu Gly Ile Ser Pro Phe Glu Val His Ala Trp Leu Leu Pro Lys  
115 120 125

gac cta ttg ctt gag tac gtg att ggt cac atg ggt cag cac acc ggc			432
Asp Leu Leu Leu Glu Tyr Val Ile Gly His Met Gly Gln His Thr Gly			
130	135	140	
gcg agc ggg agc gac act gcg tgg ctg ggg ttc cca gcg gac gag ccg			480
Ala Ser Gly Ser Asp Thr Ala Trp Leu Gly Phe Pro Ala Asp Glu Pro			
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tat gac tgg atg cgc cct ttc gga ggt cgc tta ggt cac gtc gaa gat			528
Tyr Asp Trp Met Arg Pro Phe Gly Arg Leu Gly His Val Glu Asp			
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Trp Val Gly Ser Pro Phe Glu Trp Ile Leu Arg Val Pro Ser Arg Thr			
35	40	45	
Lys Gly Ala Val Gly Glu Leu Leu Val Ser Glu Trp Ala Asn Ala Lys			
50	55	60	
Gly Leu Arg Val Lys Arg Ser Gly Ser Ser Asp Ala Asp Arg Val Ile			
65	70	75	80
Asn Gly His Arg Ile Glu Ile Lys Met Ser Thr Leu Trp Lys Ser Gly			
85	90	95	
Gly Phe Lys Phe Gln Gln Ile Arg Asp Gln Glu Tyr Asp Phe Cys Leu			
100	105	110	
Cys Leu Gly Ile Ser Pro Phe Glu Val His Ala Trp Leu Leu Pro Lys			
115	120	125	

Asp Leu Leu Leu Glu Tyr Val Ile Gly His Met Gly Gln His Thr Gly  
130 135 140

Ala Ser Gly Ser Asp Thr Ala Trp Leu Gly Phe Pro Ala Asp Glu Pro  
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Leu Leu Leu Ala Ala Gly Pro Gly Pro Tyr  
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<213> Escherichia coli

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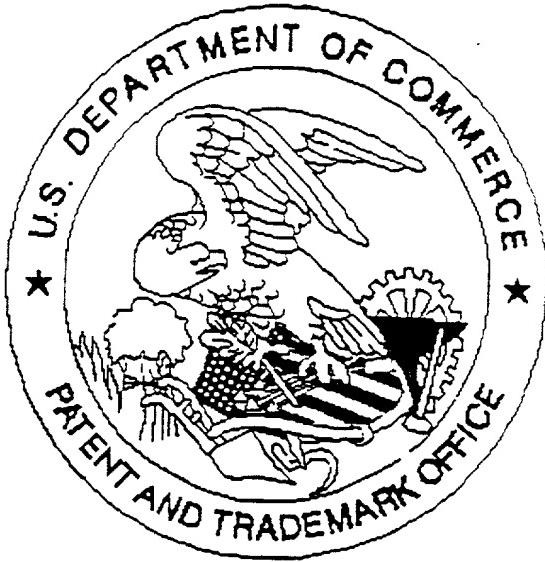
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